Fluid production and cellular elemental composition of locusta migratoria L. Malpighian tubules: a study using inhibitors and stimulators of fluid production

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Fluid production and cellular elemental composition of *Locusta migratoria* L. Malpighian tubules; a study using inhibitors and stimulators of fluid production.

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Abstract.

The *in vitro* rate and cationic composition of the fluid secreted by the Malpighian tubules of the African migratory locust, *Locusta migratoria migratoroides* L., was investigated in this study. The concentrations of the elements Na, K, P, S, Cl, Mg and Ca within *Locusta* Malpighian tubule type I cells, and the surrounding basement membrane, were quantified. Inhibitors and stimulators of fluid production were used to perturb the normal secretory state of the tubule cells.

The rate of fluid secretion under control conditions was between 1.82 and 1.33. nl min^{-1}. The fluid $[K^+]$ was approximately 126mM, and $[Na^+]$ 51mM. The basement membrane was characterised by high [Na] and [Cl] whilst a gradient of $[K]_i$ was observed. $[K]_i$ rose from approximately 193 mmol Kg^{-1} d.w. at the basal infoldings to 481 mmol Kg^{-1} d.w. at the apical microvillar border. The central cytoplasmic $[K]_i$ was 348 mmol Kg^{-1} d.w., estimated as 116mM. $[Na]_i$ and $[Cl]_i$ were generally lower, being 57mM and 29mM respectively in the central cytoplasm. Only K assumed a concentration gradient. Intracellular mass dense concretions were observed. Three types were present, the first rich in P and Ca, the second, rich in S, Na and K, and the third, rich in Mg, K and Na.

The fluid production inhibitors furosemide (1mM) and bafilomycin A_1 (1μM) raised the $[Na^+]$ in the secreted fluid, and altered $[K]_i$, $[Na]_i$ and $[Cl]_i$. Furosemide lowered $[K]_i$ but increased $[Na]_i$ and $[Cl]_i$. Bafilomycin lowered $[K^+]$ in the secreted fluid, though $[K]_i$ increased. Both inhibitors abolished the $[K]_i$ gradient. Replacing K^+ with Rb^+ in the bathing saline slowed fluid secretion and lowered $[K]_i$ and $[Cl]_i$, though a gradient of $[K]_i$ was retained. Rb adopted an intracellular gradient which mirrored that of K. Rate of secretion data suggests Rb enters the cell basolaterally primarily via the Na^+-K^+-ATPase. The fluid secretion stimulator cAMP (1mM) lowered $[K]_i$, and raised $[Na]_i$ and $[Cl]_i$, but corpora cardiaca extract left these elements' concentrations largely unchanged. Stimulation with both corpora cardiaca extract and cAMP maintained the $[K]_i$ gradient. These stimulators changed the content and number of mass dense concretions present, in manner which suggested that these structures were important in ion transport. These findings support the current model of ionic transport in these cells, including the basolateral presence of an Na^+-K^+-$2Cl^-$ cotransporter, and an apical proton-pumping V-type ATPase / K^+/nH^+ antiport complex.

"I ... had ambitions not only to go farther than anyone had done before, but as far as it was possible for man to go...."

James Cook.

"La fixité du milieu intérieur est la condition de la vie libre."

Claude Bernard.

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Declaration.

I hereby affirm that the work described in this thesis was performed entirely by the author and that no part of this work has been previously submitted for a degree in this or any other university.

For my family, with love.

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Glossary.

AAS	Atomic absorption spectrophotometry
ADH	Anti-diuretic hormone
ADP	Adenosine diphosphate
AKH	Adipokinetic hormone
AMV	Apical microvilli
ATP	Adenosine triphosphate
BA	Basement membrane
BMV	Basal infoldings
BSA	Bovine serum albumin
C	Coulombs
cAMP	cyclic adenosine monophosphate
C.C.	Corpora cardiaca
cGMP	cyclic guanosine monophosphate
CNS	Central nervous system
CRF	Corticotrophin releasing factor
CYT	Cytoplasm
DB	Dark body
DB-cAMP	Dibutyryl cyclic adenosine monophosphate
D.H.	Diuretic hormone
DMSO	Dimethyl sulphoxide
DP1	Diuretic peptide one
DP2	Diuretic peptide two
d.w.	Dry weight
E_c	Critical excitation potential
E_o	Incident radiation energy
ECF	Extracellular fluid
EDS	Energy dispersive spectrometry
E_K	Potassium equilibrium potential
EM	Electron microscope
Et-OH	Ethanol
FET	Field effect transistor
FLS	Filtered least squares

FST	Standard correction factor
FWHM	Full width of a characteristic peak at half the maximum height
GCAM	Goblet cell apical membrane
5-HT	5-hydroxytryptamine
HEPES	4-(2-hydroxylethyl)-1-piperazine-ethanesulphonic acid
IP_3	Inositol 1,4,5-triphosphate
ISM	Ion selective microelectrodes
K	Kelvin
$[K]_i$	Intracellular potassium concentration
kDa	kilodaltons
KS	Kolmogorov-Smirnov
kV	kilovolts
keV	kiloelectronvolts
LK8	Leucokinin VIII
12L:12D	12 hours light:12 hours dark light cycle
Locusta-DP	*Locusta*-diuretic peptide
Lom-MIP	Locustamyoinhibiting peptide
Lom TK I	Locustatachykinin I
Lom TK II	Locustatachykinin II
M	molar
Me^+	Monovalent metal alkali cation
mM	millimolar
mmol l^{-1}	millimoles per litre
M_r	Molecular mass
mV	millivolts
$[Na]_i$	Intracellular sodium concentration
Na^+-K^+-ATPase	Na^+-K^+-adenosine triphosphatase
NBD-Cl	7-chloro-4-nitrobenz-2-oxa-1,3-diazole
NEM	n-ethyl maleimide
nm	nanometres
NO	Nitric oxide
P	Probability
PDE	Phosphodiesterase
PKA	Protein kinase A

PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
ppm	Parts per million
P-type ATPase	Phosphorylated-type adenosine triphosphate
R1	Rate one
R2	Rate two
RER	Rough endoplasmic reticulum
revs/min	Revolutions per minute
SEM	Scanning electron microscope / standard error of the mean
STEM	Scanning transmission electron microscope
TEM	Transmission electron microscope
TEP	Transepithelial potential
t	t-statistic
V_a	Apical membrane potential
V_b	Basal membrane potential
V-type ATPase	Vacuolar-type adenosine triphosphatase
W	Watts
WDS	Wavelength dispersive spectrometry
Z	Atomic number / Z-statistic

Chapter One.

Introduction.

Bernard was the first to recognise that multicellular organisms possess their own internal bathing medium, the "milleu intérieur" (Bernard 1878). Beyenbach (1993) has drawn a satisfying analogy between the milleu intérieur (or extracellular fluid, the ECF) and a space suit - both provide their occupants with constant, protective surroundings. It is almost axiomatic that such constancy necessitates regulation, and it is not surprising that insects have evolved an array of efficient homeostatic mechanisms serving their ECF. The haemolymph experiences greater changes in composition than the vertebrate ECF during ontogeny and feeding (Beyenbach 1993). Nevertheless, experimental evidence indicates that insects are capable of efficient regulation of their ECF. This regulation of the haemolymph in insects is a primary function of the Malpighian tubules which, unlike the vertebrate kidney nephron, does not rely on pressure filtration to produce primary urine. The production of primary urine (or tubule fluid) in insects requires the active secretion of ions, and is performed by the Malpighian tubule, with subsequent "downstream" reabsorption of solutes and water in the hindgut, as may be necessary for homeostasis (Philips 1981). The exact operation of the excretory system is adapted to the diet of the insect, therefore differences exist between insect species, notably between plant (phytophagus) and blood-feeding (haematophagus) species (Zieske 1992). Whilst the majority of insects' tubule fluid production is K^+-driven, the periodic blood-meals taken by females of haematophagus species (e.g. *Rhodnius prolixus* and *Glossina* sp.) has resulted in varying dependence upon active Na^+ secretion (Philips 1981). Nonetheless, the primary role for K^+ is viewed as an adaptation to the potassium-rich plant diet of most species, but the blood feeding habit results in a greater rate of stimulated tubule fluid and urine production in haematophagus species (Philips 1981). Most insect species Malpighian tubules produce a fluid which is isosmotic to the bathing medium (*in vivo*, the haemolymph) but differs ionically from it, though the rectum alters the

composition of this fluid to maintain ECF homeostasis (Bradley 1985). This is the system present in the subject of this study, the orthopteran *Locusta migratoria migratoroides*.

First, it is appropriate to describe the structure of the Malpighian tubule. The tubules are simple, one cell thick tubular epithelia structures. Different species have different numbers of Malpighian tubules, usually arranged radially around, and emanating from, the midgut. Adults of the species *Schistocerca gregaria* (the Desert locust) have between 220 and 300 tubules in 12 groups (Savage 1956); *Locusta migratoria migratoroides* (the African migratory locust) has approximately 200 to 300 tubules, also in groups of twelve (Albrecht 1953). In the latter species, each tubule is generally approximately 15 mm long with a diameter of 50 to 80 µm, and is composed of a single layer of epithelial cells which enclose a central lumen (Bell & Anstee 1977). The proximal end of the tubules are attached to the midgut by irregularly shaped ampullae in *Locusta*, but the distal ends are blind-ending and float freely in the animal's haemocoel (Bell & Anstee 1977). Some species possess a cryptonephridial system in which the tubules lie in close proximity to the external wall of the rectum, for example *Calpodes ethlius* and *Tenebrio molitor* (Bradley 1985). The function of the cryptonephridial system varies between species, but includes the uptake of water from the atmosphere and may also encompass the additional retrieval of ions from the excretory fluid (Bradley 1985). In *Locusta*, the ampullae drain tubule fluid into the alimentary canal at the junction of the midgut and hindgut (Bell & Anstee 1977). This general arrangement of Malpighian tubules is common amongst species of, for example, Acridoidea and Blattoidea, though some other species have substantially fewer tubules. The adult dipteran *Aedes aegypti* (the yellow fever mosquito) has only five, and the Aphidoidea lack tubules entirely (Beyenbach *et al.* 1993). Pairs of parallel, helically-wound muscle fibres surround the organs and facilitate rhythmic movements of the tubules, which are readily observed *in vitro* experimental preparations of the tubules. Recent work suggests certain diuretic peptides are able to increase the contractile activity of these muscles (Coast 1998). An extensive tracheal system provides *Locusta* tubules with oxygen. Large trachea with a diameter of approximately 200 µm branch into tracheoles which wind around the surface of the tubules, also in an helical manner (Bell & Anstee 1977).

The ultrastructural features of *Locusta* Malpighian tubule cells are characteristic of many secretory epithelia. Two types of cell have been recognised in the Malpighian tubules of numerous insect species (e.g. Taylor 1971a,b; Wall *et al.* 1975), including *L. migratoria* (Peacock 1975; Charnley 1975; Bell 1977) The so-called Type I cells are in the majority, with Type II cells making up 10% or fewer of this total (Bell 1977). This secondary cell type is richly supplied with rough endoplasmic reticulum (RER) and is thought to be responsible for the secretion of mucus (Taylor 1971a,b). The Type I cell is considered to be the chief fluid secreting type and the following ultrastructural description therefore refers to this cell type.

The tubule is surrounded by a basement membrane composed of a glycosaminoglycan matrix, the major proteins being type VI collagen and laminin (Bradley 1985; Fransson 1987). The chief proteoglycan is heparan sulphate (Fransson 1987). It has been suggested that in *Locusta*, the polyanionic nature of the basement membrane may cause potassium to be concentrated at this site (Pivovarova *et al.* 1994a). The main role of the basement membrane is considered to be that of a filter, restricting access to the cell surface by haemolymph solutes and cells (Bradley 1985). Resting upon the basement membrane, the basal plasma membrane shows extensive infoldings with long extracellular channels orientated perpendicularly to the basement membrane. The large surface area available emphasises the importance of the cell in water and solute transport, and a cytoskeletal network supports the infolding architecture (Bradley 1985). Hemidesmosomes may link the basal and basement membranes (Bell & Anstee 1977; Bradley 1985). Mitochondria are present between the extracellular infoldings, and throughout the cytoplasm. Transmission electron micrographs clearly show the diverse content of the cytoplasm, including the nucleus, numerous mitochondria, endoplasmic reticulum, multivesicular bodies, lysosomes and often large numbers of electron dense granules or concretions (Bell & Anstee 1977). These mineralised concretions are a common feature of the cytoplasm of Malpighian tubule cells in many insect species (e.g. Wigglesworth and Salpeter 1969; Wall *et al.* 1975; Bradley *et al.* 1982) and exhibit a wide range of morphologies. Their function has been variously described as being that of storage excretion or as an additional store of, or indeed a transport mechanism for, intracellular cations (Pivovarova *et al.* 1994a; Wessing and Zierold 1996). They may be located in vesicles derived from the

Golgi complex or RER (Bradley 1985; Wessing & Zierold 1996). In *Drosophila hydei* larval Malpighian tubules, concretions are found in the tubule lumen (Wessing *et al.* 1992).

The lateral plasma membranes are relatively straight, and adjacent cells are joined by extensive regions of septate junctions (Bell & Anstee 1977). The apical membrane is composed of numerous tightly packed microvilli which extend into the tubule lumen, their tips displaying a somewhat bulbous morphology and containing mitochondria (Bell & Anstee 1977). Some of the microvilli share a common stem. Additionally, the apical region of neighbouring cells are linked by maculae adherens (Bell & Anstee 1977). Tubules of some insect species show both structural and functional segmentation along their length, for example in *Rhodnius* the distal portion is responsible for the secretion of a K-rich fluid, which undergoes reabsorption of water and sodium in the lower part of the tubule (Ramsay 1951). The black field cricket (*Teleogryllus oceanicus*) also displays morphological and functional difference between the distal and main segments of its Malpighian tubules (Marshall *et al.* 1993).

The ontenogenic development of the tubules is as follows. In *Schistocerca gregaria* tubules develop in 7 stages, with two groups developing during embryogenesis so that on hatching, each nymph possesses exactly 18 tubules (Savage 1956). Subsequently, 5 more groups are added, one during each instar. At the start of each stadium, new tubules begin to bud from the midgut region and undergo extensive mitosis during that instar. At the following ecdysis, this division ceases and when the next stadium begins, the newly formed cells enlarge continuously until the adult stage is reached. The maximum mean number of new tubules are added during the third instar (Savage 1956). Malpighian tubules are considered to be endodermal in origin, in contrast to the hindgut which is ectodermal and thus lined with cuticle. In the newly emerged *Schistocerca* nymph, the tubules were able to function even before they reached their full size (Savage 1956). In *Drosophila*, a special cell is present at the tip of each embryonic tubule, which promotes mitosis and is essential for the full development of the tubule (Skaer 1989; Wilkins 1995). For a review of Malpighian tubule development, see Skaer (1993).

The functioning of the Malpighian tubule cell as part of a secretory epithelium is particularly dependent on the asymmetry which exists between the apical and basolateral membranes. The polarised distribution of ion pumps,

symporters, antiporters and ion channels between these two surfaces of epithelial cells is responsible for the vectorial movement of ions, which ultimately facilitates fluid production in Malpighian tubules (Cereijido *et al.* 1989). The tubule cells have been shown to be capable of clearing a variety of substances from the haemolymph (or bathing medium). Thus, the production of a KCl-rich tubule fluid enables the passive diffusion of haemolymph solutes such as amino acids (Maddrell and Gardiner 1980a), monosaccherides, disaccherides and urea into the tubule lumen, though this is relatively slow (Philips 1981; Maddrell 1981). The permeability of the Malpighian tubules permits the automatic removal of low molecular weight waste products and toxins by passive diffusion (Ramsay 1958). To compensate for the slow clearance rate, the tubule has at its disposal a vast surface area for transport, and for some compounds, for example alkaloids (Maddrell & Gardiner 1976), sulphate ions (Maddrell & Philips 1975), acylamides, sulphonates (Maddrell *et al.* 1974) and glycosides, specific active transport mechanisms exist to speed their excretion (Maddrell 1981). In *Locusta migratoria*, active reabsorption of passively secreted sugars such as glucose and trehalose also takes place in the Malpighian tubules (Rafaeli-Bernstein and Mordue 1979). The development of such a system has been viewed as a response to the ability of insects to withstand substantial changes in their haemolymph content, aided by the development of special epithelia with regulatory properties that protect sensitive tissues (Maddrell 1981). Further advantages include the need for relatively slow reabsorption of essential haemolymph solutes "downstream" from the Malpighian tubules, and the use of the haemolymph as a storage site for sugars and lipids used as fuel during flight (Maddrell 1981).

The Malpighian tubules are not the only organs involved in the regulation of the composition of the ECF. Regions of the alimentary canal "downstream" from the Malpighian tubules are critical in the integrated functioning of the insect excretory system (Bradley 1985). Excretion of the tubule fluid as the final product of the excretory process is unlikely, since this would result in the loss of crucial cations and anions, and water (Bradley 1985). Extremely efficient water retention is a feature of many insect species, as water is reabsorbed across the cuticle-lined hindgut; and it is thought that this cuticular layer protects the cells of the rectum from the potentially damaging luminal compounds (Maddrell & Gardiner 1980b; Maddrell 1981).The fate of water, ions and various urine solutes after the primary urine has left the proximal part of the tubule has been studied at length in

phytophagus species such as *S. gregaria* (Philips 1964a,b,c) and *Periplanta americana* Malpighian tubule (Wall, 1970, 1971). A detailed description of the transport processes occurring in the ileum and rectum will not be given here, the field has been reviewed by Hanrahan and Philips (1983) and Bradley (1985). Nevertheless, in brief, as the primary urine exits the proximal region of the tubules of *Schistocerca gregaria* it enters the ileum, where its composition remains essentially unchanged (Maddrell and Klunsuwan 1973). The fluid then moves posteriorly into the cuticle-lined rectum where Na^+, K^+ and Cl^- are actively transported into the rectal pad cells. Water uptake occurs from the fluid even in the absence of these ions, via the hyperosmotic intercellular spaces, and the composition of these spaces is controlled by the recycling of ions across the lateral plasma membranes (Wall 1970, 1971; Gupta *et al.* 1980). Also, amino acids are actively reabsorbed in the rectal lumen (Chamberlin & Philips 1980, 1982; Bradley 1985). The final product of the excretory system of the phytophagus terrestrial insect is thus a more or less completely dry faecal pellet (Bradley 1985).

The Malpighian tubule has been intensively studied for more than 50 years. Wigglesworth made the first systematic study in the haematophagus species *Rhodnius prolixus* (Wigglesworth 1931a,b,c). He showed that after a blood meal, the insect underwent rapid diuresis which was completed between 3 to 4 hours post-feeding (Wigglesworth 1931a). The urine produced was mainly isotonic to the ingested blood meal and contained KCl and NaCl, though the proportion changed during diuresis (Wigglesworth 1931a). He reported that cell morphology differed along the length of the tubule; the end of the tubule distal to the ampullae was secretory and the proximal region reabsorptive (Wigglesworth 1931b). Ramsay and his co-workers subsequently refined this approach to measure simultaneously Na^+ and K^+ concentrations in the small volumes of haemolymph and urine available to them (Ramsay *et al.* 1953); in particular, an *in vitro* preparation of Malpighian tubules was developed to collect tubule fluid for analysis (Ramsay 1953). This paper showed that of eight species of insect examined, including *L. migratoria migratroides, Aedes* and *Rhodnius*, all had tubule fluid in which the K^+ concentration exceeded that of the haemolymph (Ramsay 1953). This in turn led to the suggestion that the accumulation of K^+ in the tubule fluid was probably by means of active transport in all insects (Ramsay 1953), explicitly extended to state that "...the secretion of potassium is the prime

mover in generating the flow of urine (*tubule fluid*) and that in consequence of this secretion conditions are created which enable water and other constituents of the urine to follow." (Ramsay 1956).

In the intervening period, considerable advances have been made in our understanding of how tubule fluid secretion occurs. It is the purpose of this chapter to summarise briefly these advances, leading to a model of fluid production which currently best explains the movement of cations and anions across the Malpighian tubule cell. The following description of the changes in our understanding of epithelial ion transport during this period will remain confined primarily to insect Malpighian tubules. Early workers in the field used the term "urine" to describe both the fluid voided from the anus, and the fluid produced by the Malpighian tubules themselves. In this study, which is concerned only with the latter secretion, "urine" has been replaced with "tubule fluid", as appropriate. Chapter one then concludes with a brief summary of the experimental objectives of this study.

Malpighian tubule fluid production.

As discussed above, Ramsay (1953) found that for several species of insect (*Locusta migratoria migratoroides*, *Dixippus morosus*, *Pieris brassicae*, *Dytiscus marginalis*, *Tenebrio molitor*, *Aedes aegypti*, *Rhodnius prolixus* and an unidentified Tabanid larva) that tubule fluid [K^+] exceeded that of the haemolymph, and that the tubule fluid [Na^+] was generally less than that of the haemolymph. This, coupled with measurements of the transepithelial potential (TEP) differences, led to the conclusions that active K^+ secretion into the lumen occurred in the majority of these species, and it was considered probable that the active transport of this cation was central to tubule fluid production in all insects (Ramsay 1953). Though Ramsay tentatively suggested that the secretion of K^+ (followed by an anion) might raise the osmotic pressure of the lumen and so promote the movement of water and sodium across the cell (Ramsay 1953), it was later found that in *D. morosus*, the tubule fluid was hypotonic to the haemolymph (Ramsay 1954). Thus, a directly osmotically linked solute-water coupling mechanism was considered improbable. Movement of water by "electro-endosmosis", making use of the TEP across the tubule was therefore suggested by Ramsay (1956). An alternative contemporary model, the "double membrane

theory" (Curran 1960) suggested cotransport of solute and water, with impetus coming from the active transport of solute across a selectively permeable barrier.

In a series of papers Maddrell established that in the bloodsucking species *Rhodnius prolixus*, rapid diuresis was brought about by the release of a diuretic hormone into the haemolymph from neurosecretory cells in a mesothoracic ganglionic mass (Maddrell 1962, 1963, 1964a,b). The response of the *in vitro* Malpighian tubules to diuretic hormone was sensitive to temperature, both in terms of the increase in the rate of secretion and the composition of the secreted fluid. The initiation of diuresis was thought to be triggered by distension of part of the midgut as consumption of a bloodmeal occurred. Earlier work by Nunez (1956) had indicated that a diuretic hormone produced in the brain of *Anisotarsus* increased water loss via the Malpighian tubules.

The physiology of *Dysdercus fasciatus* was then investigated by Berridge. He confirmed that a factor was present in the haemolymph that was responsible for an increase in the rate of secretion by isolated Malpighian tubules, and that this hormone was present in extract from various nervous tissues, particularly the median neurosecretory cells (Berridge 1965a). The rate of production of tubule fluid was seen to be inversely proportional to the osmotic pressure of the bathing medium. The secreted fluid was isotonic to the serum over a considerable range of osmotic pressures (Berridge 1965a). The main nitrogenous waste product in this species, allantoin, was found to be relatively impermeable across isolated tubules (Berridge 1965b). The secretion rate of Malpighian tubules of the cockroach *Periplanta americana* and *Carausius morosus* were also affected by nervous tissue extracts (Wall & Ralph 1964; Vietinghoff 1967). Mordue later showed that corpora cardiaca extract accelerated the clearance of amaranth and potassium urate across the Malpighian tubules of the desert locust *Schistocerca gregaria* (Mordue 1969). The storage lobes of the corpora cardiaca gland are an important site for the storage of neurohormones, and are closely associated with the aorta; additional functions include secretions related to moulting, pigmentation and maturation of the ovaries (Uvarov 1966).

Next, Berridge examined ionic transport in the Malpighian tubules of adult female *Calliphora erythrocephalea* (Berridge 1968). Here, the tubule fluid was slightly but consistently hypertonic to the bathing saline. The rate of fluid production was inversely proportional to the bathing saline [K^+], and tubule fluid production was possible in the presence of K^+ alone. Rubidium was able to

substitute for potassium efficiently with a small fall in the rate of secretion noted when Rb^+ replaced K^+ entirely in the bathing medium. If the *in vitro* bathing medium was predominantly Na^+-rich, and a relatively small proportion of the total Na^+ content was replaced by K^+, a dramatic rise in the tubule fluid [K^+] was noted and the rate of fluid secretion accelerated. Na^+ and K^+ transport were considered to be independent of each other, since when the [K^+] in the tubule fluid was much greater than its concentration in the bathing medium, the [Na^+] in the medium was of comparable concentration to the tubule fluid [K^+]. Berridge proposed that potassium secretion was achieved by the action of ion pumps on both the basal and apical surfaces. In spite of the apparent insensitivity of tubule function to 1mM ouabain, it was suggested that a Na^+-K^+ pump functioned at the basal surface, and an electrogenic K^+ pump was present apically. An intriguing effort was made to investigate possible K^+/H^+ exchange by using carbonic anhydrase inhibitors and by changing the pH of the bathing medium, but no effect was observed in either case. It was concluded that the ultrastructure of the basal and apical surfaces may have been able to support standing osmotic gradients responsible for solute-water coupling. The standing-gradient or local osmosis hypothesis was proposed by Diamond and Bossert (1967) and, briefly, hypothesised that the pumping of solutes across the basal membrane caused the extracellular channel to become hyposmotic, in turn allowing water to flow by osmosis into the basal infoldings. At the apical surface, a similar pumping of ions into the luminal spaces between the microvilli was thought to make these spaces hyperosmotic, resulting in water efflux into the lumen. Though doubts had already been voiced (Maddrell 1969), this theory of solute-water coupling remained well supported at this time (Berridge & Oschman 1969).

Rhodnius Malpighian tubules were then re-examined - Maddrell found that in contrast to *in vitro* tubule preparations from *Carausius* and *Calliphora*, tubules from this species were able to secret at normal rates in K^+-free solutions (Maddrell 1969). Unlike the majority of the species previously studied, the concentrations of Na^+ and K^+ were similar in the tubule fluid (Maddrell 1969). These findings departed from the orthodoxy of Ramsay's K^+-driven model of secretion, for a similar response to a small addition of Na^+ in a high-K^+ saline was noted in *Rhodnius* as that reported in *Calliphora* to a small addition of K^+ in high Na^+-saline (that is, the rate of secretion rapidly increased). The secretion rate was reduced considerably in low [Na^+] bathing medium. Since the tubules were

able to concentrate either Na^+ or K^+ when they were present in low concentrations in the bathing medium, even when the concentration of the other cation was much greater, separate transport routes were proposed for both cations (Maddrell 1969). Chloride was observed to be an essential anion, as its omission resulted in the virtual cessation of secretion. Again the rate of secretion was inversely proportional to the osmotic pressure of the bathing solution, with the tubule fluid being marginally hyperosmotic to the bathing medium (Maddrell 1969). The tubules had some capacity to replace potassium with ammonium ions. Various metabolic inhibitors such as cyanide, 2,4 - dinitrophenol and copper ions inhibited fluid production, though ouabain and acetazolamide were ineffective in this respect. Addition of 5-hydroxytryptamine (5-HT) stimulated fluid production. These data led Maddrell to suggest that entry across the basal membrane occurred in such a way as to be faster if both K^+ and Na^+ were present together, and that at the apical surface, an active cation pump existed which would transport whichever was the dominant cation intracellularly. As noted earlier, this differed considerably from the contemporary view of K^+-driven fluid production across such epithelia, even though the standing gradient model was considered to be supported by the data (Maddrell 1969). At the same time, Irvine (1969) reported on a clear regional variation in ion transport along the length of Malpighian tubules of the larvae of the lepidopteran *Calpodes ethlius*. The upper segment of the tubule (closest to the cryptonephridial complex) actively secreted K^+, which was reabsorbed in the lower segment, where instead Na^+ was secreted actively.

It is appropriate at his point to summarise the contemporary knowledge of tubule physiology into two models, one accounting for "classical K^+-driven" fluid production, and another for fluid production in *Rhodnius.* In both models, it was clear that a factor in the haemolymph was able to increase fluid production and the transport of dyes by the tubules, and that this diuretic hormone was derived from a variety of nervous tissues. Since the tubule fluid had been shown to be mainly either isotonic or slightly hypertonic to the bathing medium, water movement was considered to be passive, following the transport of cations according to the standing gradient model. Berridge's 1968 *Calliphora* model and Maddrell's 1969 *Rhodnius* model are summarised overleaf. The following convention will be adopted in the diagrams in this chapter.

Passive transport of solute.

↑ cAMP

Process stimulated by cAMP. Arrow reversed for inhibition by compound.

Water.

Transport of solute by symporter, antiporter or active transport.

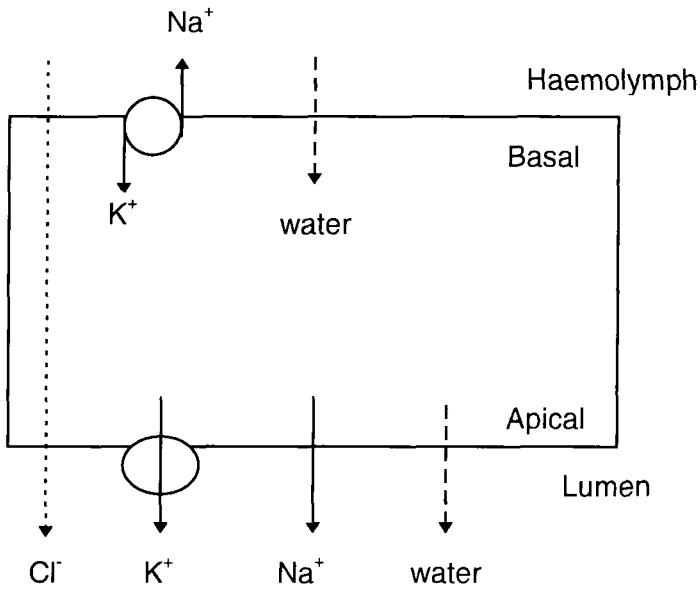


Figure 1.1. Ion transport in Berridge's *Calliphora* model (1968). Note passive transcellular movement of Cl^-.

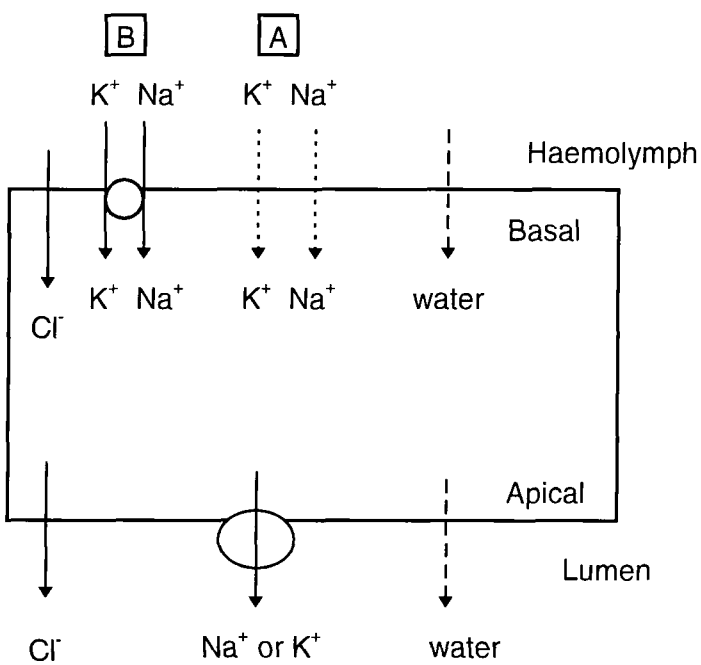


Figure 1.2. Ion transport in Maddrell's *Rhodnius* model (1969). In the absence of the other cation, Na^+ or K^+ were considered to pass passively across the basal surface (A). Together, their influx was synergistic by way of an undefined pump (B). The proposed common cation pump was present apically (Na^+-preferential).

Berridge's model (Figure 1.1) included a Na^+-K^+-ATPase at the basal surface, supplying K^+ for active apical electrogenic transport and enabling the osmotically linked movement of water across both plasma membranes. Apical transport of Na^+ was considered to be independent of K^+. It was suggested that chloride movement was as the result of an electrical gradient created by the net active transport of potassium (Berridge 1968).

The model proposed for *Rhodnius* differed considerably (Figure 1.2) (Maddrell 1969). At the basal surface, when either potassium of sodium were present alone, it was postulated that they entered the cell by diffusion. When present together, an unknown mechanism was proposed to accelerate synergistically the entry of both. The common cation pump shown on the apical surface was considered to be active. It was thought that active transport of chloride also occurred on the basis of measured transepithelial potential differences (Maddrell 1972). *Rhodnius* was seen to be particularly sensitive to the concentration of Cl^- in the bathing fluid (Maddrell 1972). The apparent difference in the mechanisms of fluid production in the two species was attributed by Maddrell to the function of the tubules in *Rhodnius* (Maddrell 1977; Bradley 1985). The irregular ingestion of bloodmeals by *Rhodnius* necessitates rapid diuresis followed by long periods of much slower secretion by the tubules, in contrast to phytophagus species. Since blood meals are rich in Na^+ it would be of adaptive advantage to utilise this cation as the prime mover for post-prandial secretion(Maddrell 1977). Although this second model appeared less complete than the first, it had been shown for the first time that fluid secretion could be speeded in the presence of a non-nervous tissue extract in both *Rhodnius* and *Carausius* , 5-hydroxytryptamine (5-HT) (Maddrell *et al.* 1969). The model did not exclude the standing gradient model on a local scale, that is within the basal infoldings or luminal microvillar channels of a single cell, but the theory that such gradients occurred transversely along the length of the lumen was rejected since the tubule fluid was uniformly isotonic to the bathing medium (Maddrell 1969).

Following the finding that 5-HT was stimulatory to Malpighian tubule fluid secretion, Maddrell and co-workers investigated the pharmacological properties of a considerable number of tryptamine compounds (Maddrell *et al.* 1971). At the time, though 5-HT increased the rate of secretion of *in vitro* tubule preparations from *Rhodnius* and *Carausius*, it was not considered to be the *in vivo* diuretic hormone. The application of cyclic AMP (cAMP) also stimulated the rate of fluid

production in both species. Although the phosphodiesterase (PDE) inhibitor aminophylline had a non-stimulatory effect upon *Rhodnius* secretion, the opposite was observed in *Carausius*. These data were considered to support the hypothesis that basal cell surface receptors interacted with 5-HT, diuretic hormone and other stimulant molecules, to increase secretion via the action of adenylate cyclase and cAMP, possibly via an increase in the ion permeability of the cell (Maddrell *et al.* 1971).

cAMP also stimulated the production of fluid by *in vitro* tubule preparations of *Schistocerca gregaria* (Maddrell & Klunsuwan 1973). This study confirmed that *Schistocerca* was a typical "K^+-secretor", i.e. the rate of secretion was proportional to the bathing medium [K^+] and inversely proportional to the osmotic pressure of the bathing medium. The fluid was isosmotic to the bathing medium, and exhibited an elevated [K^+] (Maddrell & Klunsuwan 1973). The need for active K^+ transport against a concentration and electrical gradient was clear from the recorded TEP of +16.6 mV (lumen positive). 5-HT did not raise the rate of secretion, but even so, it seemed likely that fluid production was under the control of a hormone stimulated / cAMP-mediated system (Maddrell & Klunsuwan 1973). Later, Aston (1975) proved that an *in vivo* increase in $[cAMP]_i$ accompanied stimulation of fluid secretion in *Rhodnius* by diuretic hormone. The PDE inhibitor theophylline prevented the gradual decrease in $[cAMP]_i$ noted over time in control tissue, further supporting the role of intracellular cAMP as a second messenger for diuretic hormone. It therefore appeared that the hormonal stimulation previously seen to increase fluid production acted via cell membrane receptors and the intracellular second messenger cAMP in *Rhodnius* and *Schistocerca*. The route by which this second messenger affected the movement of ions was unknown.

On the basis of data collected from *Glossina austeni* and *Glossina morsitans*, a model of ion transport was proposed in these haematophagus species (Gee 1976a), see Figure 1.3. Unlike *Rhodnius*, *Glossina* was unable to secrete in K^+-free saline (Gee 1976a), and the K^+ concentration in the tubule fluid during stimulated fluid production was very much lower than then seen in *Rhodnius* (Gee 1975a). The tubule fluid was isosmotic to the bloodmeal, and the rate of fluid production was proportional to the [Na^+] in the meal (Gee 1975a, 1976a). Only Li^+ and NH_4^+ were able to replace Na^+ to any extent. *In vivo* fluid production was under neurohormonal control as in other species (Gee

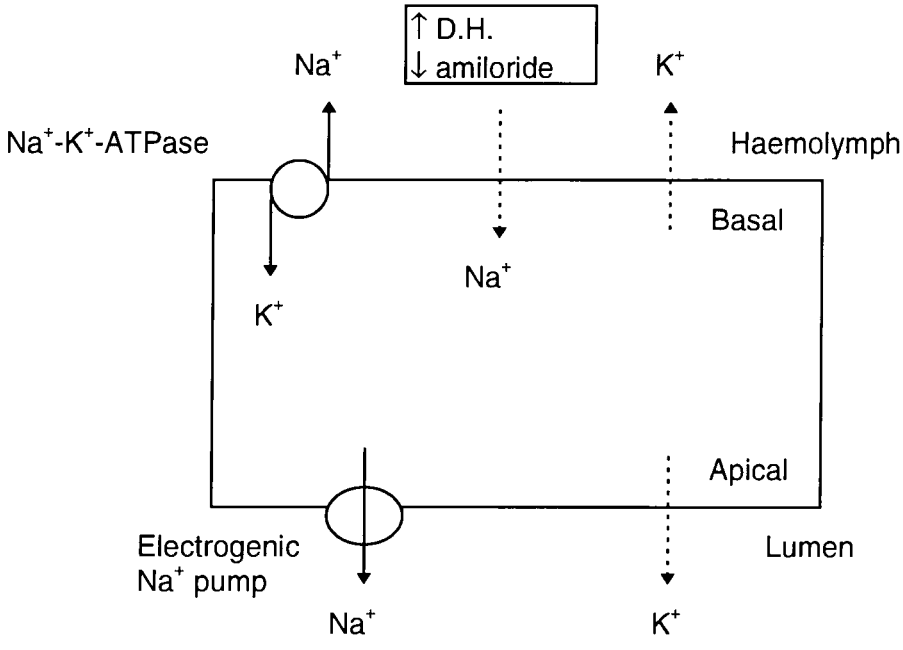


Figure 1.3. Ion transport in the *Glossina* model. Diuretic hormone (D.H.) was though to increase basal permeability to Na^+, whilst amiloride decreased it. After Gee (1976a).

1975b). *Glossina* was well adapted to the blood-feeding diet. *Rhodnius* was considered to represent a species in which post-prandial diuresis was chiefly driven by Na^+, though K^+ was also actively transported, yet in *Glossina* active transport of K^+ probably did not occur (Gee 1976a). The stated model, despite the ouabain insensitivity of the tubule preparations, relied heavily upon a basal Na^+-K^+-ATPase to retain the presence of low $[Na^+]_i$ and high $[K^+]_i$, hence the requirement for K^+ extracellularly to maintain fluid secretion (Gee 1976a). In consequence of the action of the ATPase, the entry of Na^+ basally was thought to be passive down its concentration gradient, activating the ouabain-insensitive apical pump. Basal permeability to Na^+ was thought to be increased by diuretic hormone. The inhibitory effect of amiloride and ethacrynic acid on the secretion rate was taken to further support the passive permeability of the basal membrane to Na^+, and the non-involvement of the basal ATPase in fluid secretion (Gee 1976b).

Until this point in time, the methods available for investigating ion and water transport in Malpighian tubule tissue and other secretory and absorptive epithelia were restricted to measuring the rate and composition of the fluid produced, and *in vitro* electrophysiological measurements from tubule cells. The data derived from these techniques were limited. Then, Gupta *et al.* (1976) applied electron probe X-ray microanalysis to the measurement of elemental concentrations intracellularly and within extracellular spaces in the upper segment of *Rhodnius* Malpighian tubule. It was shown for the first time that the distribution of elements within a secretory epithelial cell were not uniform, but that intracellular gradients of Na, K and to a lesser extent Cl, existed (Gupta *et al.* 1976). From the increase in both $[Na]_i$ and sodium concentration in the fluid when the tubules were stimulated by 5-HT, it was assumed that this compound caused a rapid influx of Na^+ across the basal membrane to supply the previously hypothesised common cation pump and increase fluid secretion. Measurements taken from the luminal brush border extracellular spaces proved that gradients of Na, K and Cl were present, but since their concentrations increased the closer probe measurements were recorded towards the main luminal space, the results were inconsistent with the Diamond and Bossert standing gradient model of solute-water coupling (Diamond & Bossert 1967). Finally, the noted anion deficit at the apical brush border and the high [K] at the basement membrane were both

proposed to be due to the binding of cations at these sites to anionic regions of proteoglycans or collagen (Gupta *et al.* 1976).

These investigations were extended to include other secretory and absorptive epithelia, including *Calliphora* salivary glands (Gupta *et al.* 1978), rectal papillae (Gupta *et al.* 1980) and rabbit ileum (Gupta *et al.* 1978). These studies suggested that for both secretory and absorptive epithelia, the elemental concentrations in the extracellular spaces resulted in the development of a hyperosmotic regions into which water was thought to flow by osmosis, down so-called "local osmotic gradients". In this way, ion transport was coupled to fluid flow, though the assumption in previous models (Diamond & Bossert 1967; Hill 1975a, 1977) that water flow was exclusively transcellular was questioned (Gupta *et al.* 1978a). It was suggested that at least some water flow was paracellular, in the intercellular spaces between adjacent cells (Gupta *et al.* 1978a). In no instance did the data support the standing gradient model, supporting the earlier assertion that the apical extracellular channels were too short to develop the required gradient (Taylor 1971a). An alternative model was available; Hill (1975a,b) described electro-osmosis, in which the apical membrane potential difference (generated by the electrogenic cation pump) caused passive Cl^- efflux into the lumen, in turn facilitating the "dragging out" of water via frictional interactions with the anions (Maddrell 1977). Ion-selective microelectrodes (ISM) were used to validate electron microprobe results from *Calliphora* salivary gland (Gupta *et al.* 1978a). From the ISM results, it was suggested that nearly all the elemental K measured by X-ray microanalysis was "free", i.e. existed in the cationic form. A large proportion of the chloride was thought to be bound, with at least 30% of the measured elemental Na similarly bound or compartmentalised (Gupta *et al.* 1978a). A model for fluid secretion by *Calliphora* salivary glands was proposed Gupta *et al.* (1976). Figure 1.4 shows a diagram of this model. Concisely, a non-specific apical cation pump was believed to be present, whose activation was mediated through the intracellular second messenger cAMP, when the cells were stimulated by 5-HT (Berridge & Prince 1972). The pump was able to substitute K^+ with either Rb^+ or Na^+ (Berridge *et al.* 1976), to the extent that

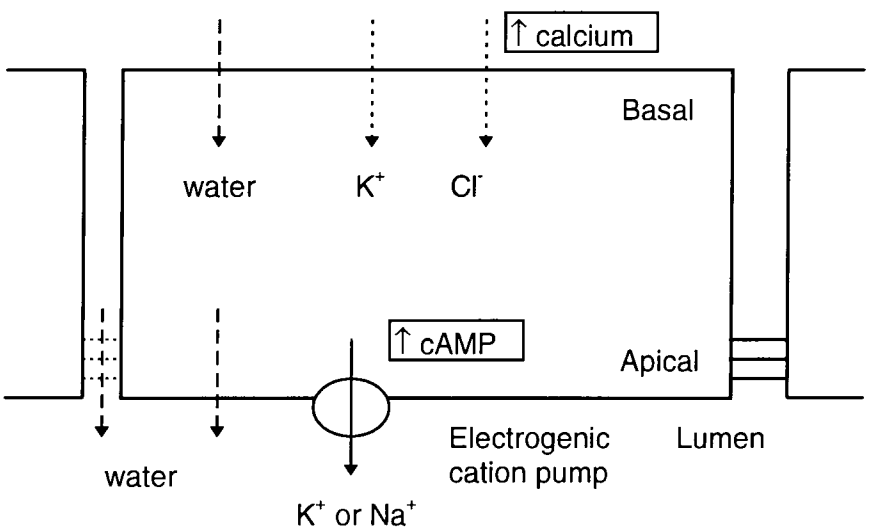


Figure 1.4. Ion and water transport in *Calliphora* salivary gland (after Gupta *et al.* 1978). Note possible paracellular movement of water.

the cell could almost entirely replace K^+ with Na^+ intracellularly and hence secrete Na-rich fluid instead (Gupta *et al.* 1978). The influx of K^+ normally required for fluid production was considered to occur through passive influx down an electrochemical gradient initiated by hyperpolarisation of V_b (Berridge & Schlue 1977). This hyperpolarisation was thought to be the result of the efflux of Cl^- as it followed K^+ across the apical membrane (Berridge *et al.* 1975). The second arm of 5-HT stimulation, via calcium, was thought to increase the basal permeability of Cl^- (Berridge *et al.* 1975). Cation extrusion into the lumen resulted in the passive efflux of Cl^- to retain electroneutrality (Prince & Berridge 1972). As well as supporting the solute-water coupling hypothesised for this tissue, X-ray microanalysis showed that the changes in $[Cl]_i$ on 5-HT stimulation were consistent with the model detailed in Figure 1.4 (Gupta *et al.* 1978). These results suggested that the ability of the proposed electrogenic apical pump to transport either Na^+ or K^+ was a general feature of this transporter in secretory epithelia.

What characterised both this *Calliphora* model and the one developed for *Rhodnius* Malpighian tubules (Maddrell 1977) was their omission of an Na^+-K^+-ATPase. The inability of many workers to demonstrate sensitivity to ouabain in Malpighian tubules was well documented (e.g. Rafaeli-Bernstein & Mordue 1978; Berridge 1968; Maddrell 1969), to the extent that *Oncopeltus fasciatus* tubules could actually concentrate this inhibitor (Maddrell 1977). The contradiction over the presence of a classical Mg^{2+}-dependent Na^+-K^+-ATPase in Malpighian tubule cells was resolved when the activity of this enzyme was demonstrated in microsomal preparations of Malpighian tubules from *Schistocerca gregaria* (Peacock *et al.* 1972) and later *Locusta migratoria* (Anstee & Bell 1975). These workers also demonstrated that ouabain inhibited fluid production of *in vitro* tubule preparations of *Locusta*, at concentrations between 10^{-3} and 10^{-6}M (Anstee & Bell 1975). Many previous studies had suggested that such secretory cells were ouabain-insensitive, but the findings of Anstee and colleagues in *Locusta* were supported by other workers (Weber-Von Grotthaus *et al.* 1974; Farquarson 1974; Gooding 1975). One reason for the ineffectiveness of ouabain was the incubation temperature used in many of the experiments as the percentage of fluid production inhibited by ouabain was undoubtedly temperature dependant (Donkin & Anstee 1980). Typically, *in vitro* preparations of Malpighian tubules were usually incubated at room temperature (apparently usually between 20-24°C), whilst Anstee *et al.* (1979) used 30±1°C. Thus, in some earlier studies,

the failure to maintain a specific temperature, and to use a more physiologically relevant temperature compromised the models of cell functioning being developed, in spite of the earlier knowledge that lowering the temperature affected both the rate and composition of the fluid produced by *Rhodnius* Malpighian tubules (Maddrell 1964a). Other causes for the reported discrepancies in the effect of ouabain on fluid production by Malpighian tubules were reviewed by Anstee and Bowler (1979).

The *in vitro* secretion rate of *Locusta* Malpighian tubules' was inhibited by ouabain (Anstee & Bell 1975) at 30±1°C and both Na^+ and K^+ were required for optimal secretion rates to be achieved (Morgan & Mordue 1981). At 1 mM, ouabain also raised the Na^+ : K^+ ratio in the fluid (Anstee *et al.* 1979). As with many other species, the fluid was slightly hyperosmotic to the bathing saline, and K^+ was secreted in preference to Na^+, even against a substantial concentration gradient (Anstee *et al.* 1979). The rate of fluid production was again inversely proportional to the osmotic pressure of the bathing saline (Anstee *et al.* 1979). Measurement of the transepithelial potential supported the presence of the Na^+-K^+-ATPase (Anstee *et al.* 1980). The same study showed that addition of between 10^{-4} and 3×10^{-3} M cAMP stimulated the secretion rate, but 5-HT did not (Anstee *et al.* 1980). cAMP hyperpolarised the lumen significantly, suggesting an influx of K^+ occurred (Anstee *et al.* 1980). This was confirmed by Morgan and Mordue in the same tissue, and a similar effect was elicited by diuretic hormone extracted from the corpora cardiaca gland (Morgan & Mordue 1981). Na^+ was shown to be essential in obtaining stimulated fluid secretion, and high [Na^+] and [Cl^-] in the bathing medium was needed for maximal secretion (Morgan & Mordue 1981).

At this time, a review appeared which summarised most of the known aspects of ion and water transport in insect epithelia (Philips 1981). Significantly, Na and Cl cotransport was proposed to occur at the basal surface of the cells (Philips 1981). The basal entry of Cl^- into the cell was against an electrical gradient and slight concentration gradient, and luminal efflux was thought to be passive (Philips 1981). A model was suggested in which Na^+ drove Cl^- into the cell via a cotransport mechanism; the cation was then recycled via the Na^+-K^+-ATPase. This model is shown in Figure 1.5. The model conveniently explained the dependence of secretion on Na^+ as well as K^+ in K^+-driven tubules, and was supported by the total inhibition of fluid production in *Rhodnius* by furosemide

(Philips 1981, quoting pers. comm. with Maddrell and O'Donnell). Furosemide, a loop diuretic, was known to be an inhibitor of NaCl cotransport in other cells, for example avian erythrocyte (Palfrey *et al.* 1980) and rat ileum (Humphries 1976). At this time a relative paucity of information on the action of diuretic hormone on ionic transport and water transport in Malpighian tubules existed (Philips 1981); only in a few cases had any effort been made to purify and characterise these factors, for example Aston (1979), Mordue & Goldsworthy (1969).

Locusta neurosecretory hormones were found to be produced in the median neurosecretory cells of the brain, then transported along nerve cell axons to the corpora cardiaca where they were stored (Morgan & Mordue 1984a). A potent peptide diuretic hormone was isolated from the storage lobes of the corpora cardiaca, and it was suggested this hormone existed in 2 forms (Morgan & Mordue 1983a). Later it was shown that the corpora cardiaca was the main site of storage of these two diuretic hormones, though at least one form was distributed throughout the CNS (Morgan & Mordue 1984a). Since *in vitro* experiments showed that relatively high doses of the hormone were needed to stimulate maximal secretion, it was speculated that this pattern of distribution *in vivo* was to facilitate enhanced fluid production when small amounts of the diuretic hormone was released from many sites (Morgan & Mordue 1984a). Unlike Anstee *et al.* (1980) they also showed that 5-HT stimulated *in vitro* fluid secretion, but not through the action of the second messenger cAMP (Morgan & Mordue 1984b). Although 5-HT stimulated fluid production in *Rhodnius* (Maddrell *et al.* 1971), and caused ultrastructural changes in the tubule cells of this species (Bradley & Satir 1981), its effect in *Locusta* was disputed (Rafaeli & Mordue 1982). Crucially, as observed in *Rhodnius* (Aston 1975), it was seen that *Locusta* diuretic hormone raised $[cAMP]_i$ and activated adenylate cyclase (Morgan and Mordue 1984b). A model was presented for the stimulation of secretion by a diuretic hormone in *Locusta* (Morgan and Mordue 1984b). It was proposed that 2 receptors were present on the basal membrane. The receptors' action was proposed to be mediated by different intracellular second messengers, one arm via adenylate cyclase-cAMP and, more tentatively, the other by Ca^{2+} (Morgan & Mordue 1984b). *Calliphora* salivary gland possessed a similar receptor mechanism, one acting via cAMP and the other via elevated $[Ca^{2+}]_i$ (Berridge & Prince 1973). This model accounted for the

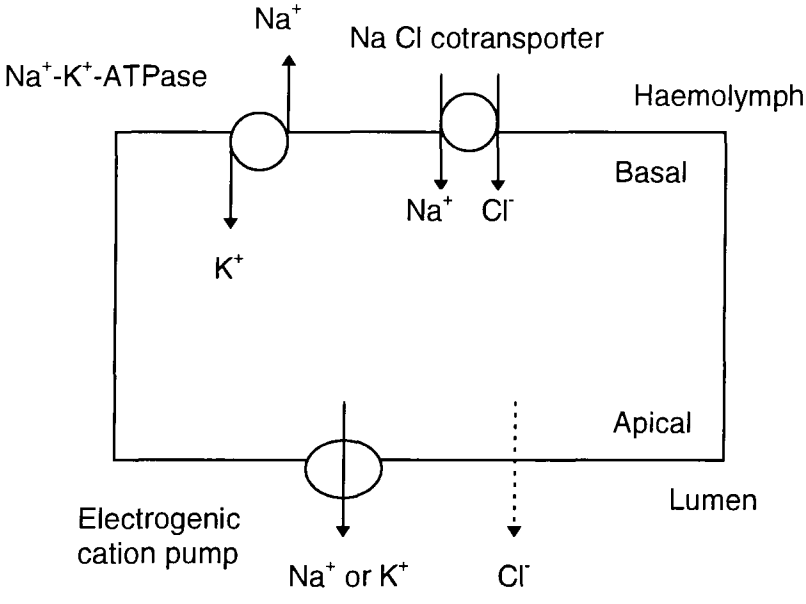


Figure 1.5. Ion transport in Philip's model (1981).

in vitro action of exogenous cAMP (Anstee *et al.* 1980; Morgan & Mordue 1984b). These workers were not able to differentiate between co-operative binding to the two sites by the same diuretic factor, and independent binding by two separate agonists (Morgan & Mordue 1984b). Adenylate cyclase-catalysed cAMP synthesis was shown to be enhanced by diuretic hormone, and the phosphodiesterase inhibitors theophylline and IBMX raised secretion rates in *Locusta* tubules (Morgan & Mordue 1985). Partial amino acid sequence data was obtained for one of the forms of diuretic hormone, and another peptide from the corpora cardiaca extract of unknown function (Morgan *et al.* 1987). It was shown that diuretic peptide 1 (DP1) exerted its action via adenylate cyclase, but DP2 site of action was unknown (Morgan *et al.* 1987).

But what was the specific site of action of cAMP? In *Aedes aegypti*, cAMP caused the tubule cell basal membrane potential (V_b) to depolarise, increasing this membrane's conductance to Na^+ (Sawyer & Beyenbach 1985). cAMP increased the production of a NaCl-rich fluid by these cells (Petzel *et al.* 1987). A very similar model to that of Morgan and Mordue was advanced, with cAMP directly increasing the basolateral Na^+ conductance; in this species, a peptide factor isolated earlier (Petzel *et al.* 1985; Petzel *et al.* 1986) from the head of the insect was able to closely mimic the action of exogenous cAMP, and hence an *in vivo* role for this peptide in cAMP-mediated stimulation was proposed. The factor was called mosquito natriuretic factor (MNF), and was considered to be the *in vivo* neurohormone (Petzel *et al.* 1987).

Additional evidence for electroneutral entry of cations and anions across the basal surface of tubule cells came from microelectrode studies in *Locusta* (Morgan & Mordue 1983b). Transepithelial potential was of similar magnitude to that found by Anstee *et al.* (1980) and Fathpour *et al.* (1983). V_b remained unchanged when either cAMP or diuretic hormone were applied to the cells (Morgan & Mordue 1983b). The basolateral membrane was permeable to K^+, since increasing $[K^+]_o$ depolarised the membrane, and decreasing $[K^+]_o$ hyperpolarised it. The $[Cl^-]_i$ was high enough to suppose an energy-consuming mechanism for basal influx, but apical efflux would have been favoured by a considerable electrical gradient. Also at the apical surface, it was clear that entry of K^+ into the lumen was against a large concentration and electrical gradient, requiring an active apical pump as previously suggested (Morgan & Mordue 1983b). Extrapolating from *Rhodnius* tubule data (Gupta *et al.* 1976) it was

supposed that Na^+ entry was passive as a consequence of a favourable electrical and concentration gradient, but that apical efflux would require active transport to overcome the substantial electrical gradient (Morgan & Mordue 1983b). A model was advanced, see Figure 1.6, in which the common cation pump was absent.

Further evidence was obtained as to the nature of basal ion fluxes in *Rhodnius*, with the finding that two fluid secretion inhibitors, the loop diuretics bumetanide and furosemide, altered the tubule TEP and V_b, but had no significant effect on V_a (O'Donnell & Maddrell 1984). Confirmation was obtained of the inhibitory effect of these compounds upon the rate of fluid secretion, and their electrophysiological effects were reminiscent of the effect of chloride-free saline (O'Donnell & Maddrell 1984). The conclusion reached was that basal Na^+ and K^+ entry was in cotransport with Cl^-, with a stoichiometry of $1Na^+:1K^+:2Cl^-$, see figure 1.7. The model embraced the common cation pump apically, required to move cations against a 35 mV electrical gradient, and the basal cotransport linked uphill Cl^- cotransport to dissipative Na^+ entry (O'Donnell & Maddrell 1984). Since the cotransporter would be *de facto* electrically neutral, this explained satisfactorily the small change elicited by 5-HT on V_b, whilst V_a and the TEP were subject to a complex triphasic change (O'Donnell & Maddrell 1984).

The presence of such Na^+-K^+-Cl^- cotransport in *Locusta* tubules was questioned by the finding that bumetanide and furosemide did not have the same effect on the membrane potentials - neither were the effects of incubation in Na^+ and Cl^- -free saline equivalent (Baldrick *et al.* 1988). Furosemide inhibited the rate of tubule fluid production in *in vitro* preparations of *Locusta* Malpighian tubules (Kalule-Sabiti 1985; Baldrick 1987), but Na^+ and Cl^- flux data argued against cotransport of these two ions across the basal membrane during inhibition with this compound (Fogg *et al.* 1993). The control membrane potential differences were such that the lumen was positive in relation to the bathing medium, with a TEP value of +5.7 mV. Both V_b and V_a were negative. It was considered likely that the Na^+-K^+-ATPase was located on the basolateral surface of the locust Malpighian tubule cell, though in spite of an electrogenic stoichiometry, it did not contribute significantly to the size of V_b (Baldrick *et al.* 1988), but clearly was important in maintaining cation transport (Fathpour *et al.* 1983).

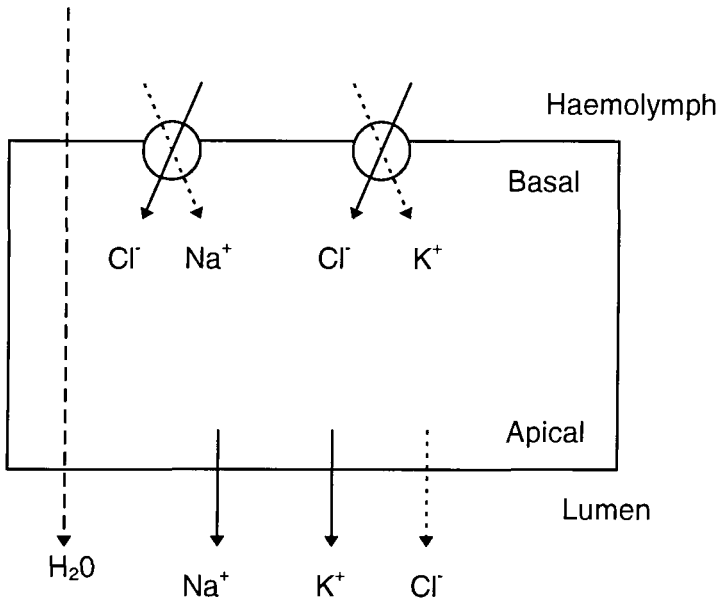


Figure 1.6. Ion transport in *Locusta* (Morgan and Mordue 1983b). Note the passive basolateral entry of Na^+ and K^+.

Na-K-Cl

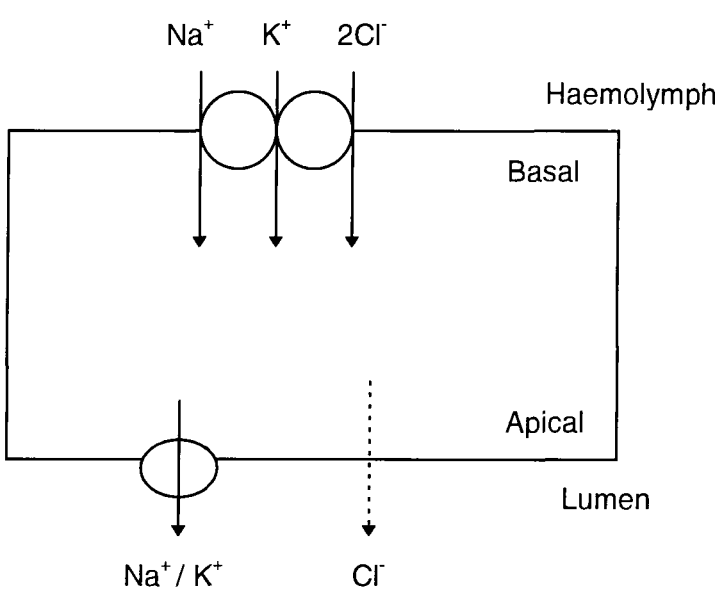


Figure 1.7. Ion transport in O'Donnell and Maddrell's *Rhodnius* model (1984). The common active cation pump was present on the apical surface.

A parallel microelectrode study on *Locusta* was performed using stimulators of fluid production (Fogg *et al.* 1989). Corpora cardiaca extract caused V_a to depolarise considerably, with a smaller but still significant hyperpolarisation of V_b, with the net result that the TEP depolarised. In contrast, cAMP, had no effect on V_b but depolarised V_a, hyperpolarising the TEP (Fogg *et al.* 1989). These results, together with those recorded from cells stimulated with IBMX, and with the stimulators in the presence of Cl^- free saline, did not exclude electroneutral cotransport at the basal surface. It seemed likely that one effect of corpora cardiaca extract was to produce an increase in $[cAMP]_i$, but that on its own, the cyclic nucleotide could not replicate exactly the same response as diuretic hormone (Fogg *et al.* 1989). Corpora cardiaca extract was also responsible for altering some aspect of chloride movement across both membranes (Fogg *et al.* 1989). The corpora cardiaca extract increased intracellular levels of cAMP, and also IP_3 (Fogg *et al.* 1990). It was proposed that cAMP acted on the apical cation pump, but Ca^{2+} increased the conductance of anions across both membranes (Fogg *et al.* 1990). It was speculated the latter conductance may have been under the control of DP2 (Morgan *et al.* 1987; Fogg *et al.* 1990). Cyclic AMP and head extract had differential effects upon the ionic character of fluid produced by the Malpighian tubule cells of *A. aegypti*, though they both increased the secretion rate by equivalent amounts (Williams & Beyenbach 1983). Electrophysiological evidence showed that head extract increased coupled cation and anion transport, that is K^+, Cl^- and probably Na^+, but it was not possible to attribute this to Na^+-K^+-Cl^- cotransport since furosemide had no effect on stimulation by the extract (Williams & Beyenbach 1984). Cyclic AMP was in contrast able to stimulate active secretion of Na^+ (Williams & Beyenbach 1984).

At this time, transport across the apical surface was still considered to be driven by a cation pump about which little was known. Then, Weiczorek and co-workers proposed that the K^+ flux across the apical membrane of the midgut goblet cell of larval *Manduca sexta* was driven by a vacuolar type ATPase (Weiczorek *et al.* 1989). Cioffi and Wolfsberger (1983) initially isolated the goblet cell apical membrane (GCAM), and ATPase activity was later documented in this tissue (Weiczorek *et al.* 1986). An ATPase with many of the characteristics of the V-type ATPase, both in functioning and the composition of subunits, was partially

purified from the apical membrane fraction of this tissue (Schweikel *et al.* 1989). This type of ATPase had hitherto been chiefly observed on the endomembranes of acidic vesicles (Klein *et al.* 1991). It was postulated that the movement of potassium ions was the result of a two component system in which the V-type ATPase generated a proton gradient across the apical membrane, energising electroneutral antiport of K^+ and H^+. An anion conductance completed the model, see Figure 1.8 (Weiczorek *et al.* 1989). It was noted that the goblet cell cavity would not necessarily be strongly acidified, and that under physiological conditions the apical membrane potential might be the main source of power for the antiporter. This model of electrogenic K^+ transport was hypothesised to occur in Malpighian tubules (Weiczorek *et al.* 1989), though the possibility that the ATPase directly transported potassium ions in the midgut epithelia could not be excluded. The sensitivity of proton transport in highly purified apical membrane preparations of the lepidopteran midgut to bafilomycin A_1, and the ATPase's independence from extravesicular $[K^+]$ later proved that it was exclusively a proton pump (Weiczorek *et al.* 1991). Bafilomycin is a highly specific inhibitor of vacuolar type ATPases (Bowman *et al.* 1988) active in the nanomolar range in *Manduca sexta* midgut (Weiczorek *et al.* 1991). The apical antiporter could be separately inhibited by using amiloride, and it was perceived to be electrogenic, recycling more than one H^+ for each K^+ extruded (Weiczorek *et al.* 1991). *Drosophila hydei* Malpighian tubule secretion rate was also inhibited by bafilomycin A_1, causing secretion to cease completely at a concentration of 10μM (Bertram *et al.* 1991). The secretion rate of both Na^+ and K^+ dropped, but the increase in Na^+ concentration in the tubule fluid was small in comparison with that observed when the inhibitors vanadate and ouabain were applied (Bertram *et al.* 1991). In view of these findings, and the earlier discovery that these cells were susceptible to inhibition by amiloride (Bertram 1989), a unified scheme for ion movement across both basal and apical surfaces was presented, shown in Figure 1.9 (Bertram *et al.* 1991). The function of the basolateral Na^+-K^+-ATPase was regarded as primarily for the maintenance of low $[Na^+]_i$ rather than for transcellular K^+ transport (Bradley 1989; Bertram *et al.* 1991). The proton gradient necessary to power the cation$^+$/H^+ antiporter was provided by an apical V-type ATPase, chiefly on the basis of the tubule's sensitivity to bafilomycin A_1; the effect of vanadate on secretion in this tissue was thought to be a result of its interaction with the basolateral Na^+-K^+-ATPase (i.e. P-type) (Bertram *et al.* 1991).

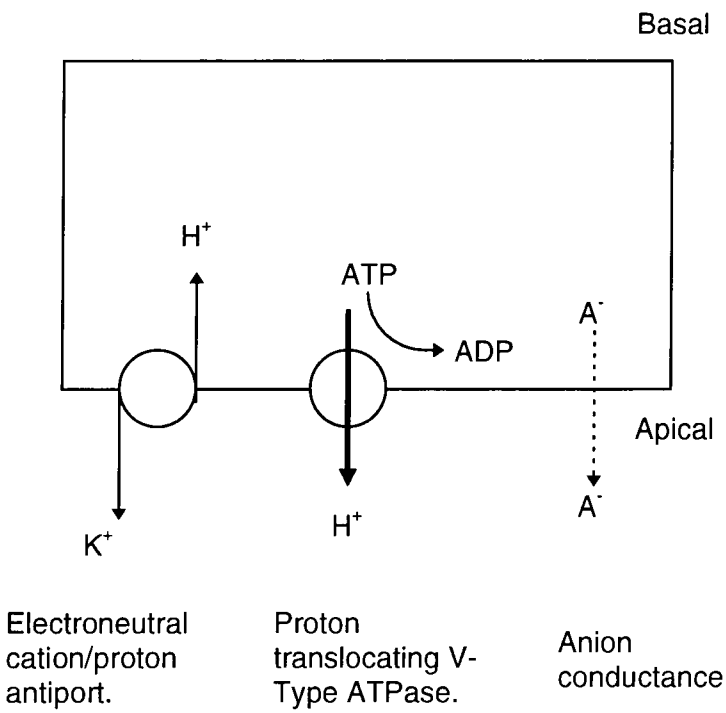


Figure 1.8. Ion transport in the *Manduca sexta* midgut model (Weiczorek *et al.* 1989). A^- represents the anion conductance.

Though an aspect of the common cation pump hypothesis was retained, in that it was thought that the antiporter might also transport Na^+ if $[Na^+]_i$ was high enough, this theory had the benefit of simplicity since it did not postulate an active cation pump able to transport 2 different cations and therefore seemingly unique to insect epithelia.

Immunocytochemical localisation of the V-type ATPase confirmed an apical location for this enzyme in *Manduca* larval midgut, and using antibodies raised to purified midgut ATPase, the Malpighian tubules from this species were probed (Klein *et al.* 1991). Both immunocytochemically stained cryosections and immunoblots supported a similar apical site for the V-type ATPase, which consisted of 6 subunits ranging in size from 67×10^{-3} M_r to 16×10^{-3} M_r in the midgut (Klein *et al.* 1991). Klein suggested that in insect epithelia as diverse as the Malpighian tubules, rectum, midgut and sensilla, the pre-eminent membrane energising enzyme ATPase was the apical V-type ATPase (Klein 1992). In light of this, Maddrell and O'Donnell (1992) described a new general model for ion transport identical at the apical surface to that in *Drosophlia* (Bertram *et al.* 1991). Inexplicably, the basal Na^+-K^+-ATPase was once more omitted from the Maddrell and O'Donnell model (Maddrell and O'Donnell 1992); correspondingly, its presence was dismissed incorrectly in *Manduca* midgut (Klein 1992). This enzyme's basolateral presence was firmly established in *Locusta* Malpighian tubules (Fogg *et al.* 1991). Cation and anion transport into the cell basally was facilitated by a Na^+-K^+-$2Cl^-$ cotransporter, and undefined additional entry sites in the form of either channels or transporters (Maddrell & O'Donnell 1992). The effect of amiloride, presumed to be specific to Na^+/H^+ antiporters, seemed to suggest that only one antiporter was present with the ability to pump both Na^+ and K^+ (Maddrell & O'Donnell 1992). However, Pivovarova *et al.* (1994b) proposed that in *Locusta* Malpighian tubules cells, K^+ exited the cell into the lumen through a different mechanism to that of Na^+. This was based on the finding that in cells incubated in Rb-containing saline (that is, without K^+), even when measurements of the $[K]_i$ by X-ray microanalysis showed that this concentration had decreased to only 10% of its normal level, K^+ was still the dominant cation in the tubule fluid though the $[Na]_i$ rose considerably (Pivovarova *et al.* 1994a). The level of Na^+ secreted remained constant in Rb-containing saline, possibly as a result of a separate efflux site, which was saturable at normal $[Na]_i$ (Pivovarova *et al.* 1994a). Moreover, Na^+-K^+-ATPase

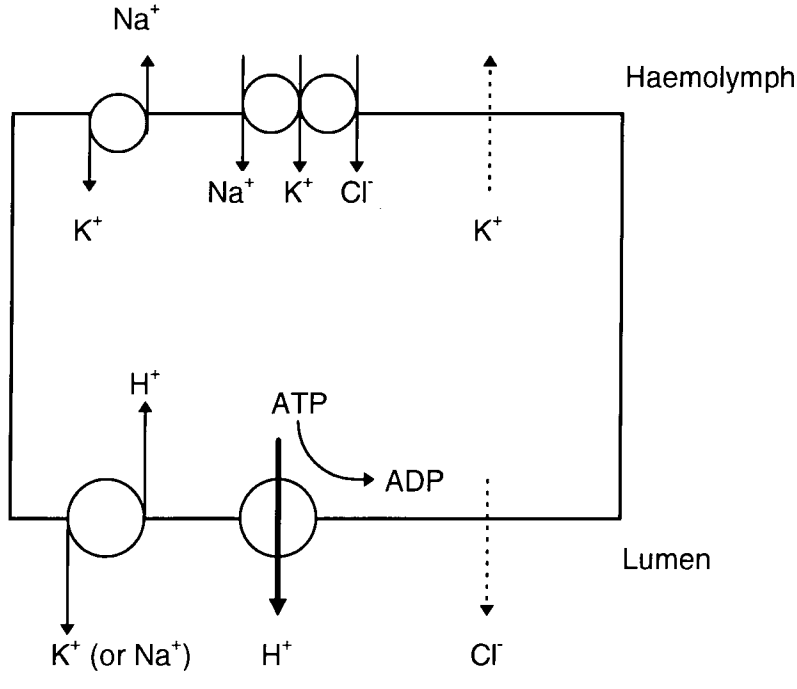


Figure 1.9. Ion transport in the *Drosophila hydei* model (Bertram *et al.* 1991).

activity was confirmed as crucial since ouabain lowered $[K^+]_i$ and raised $[Na]_i$ (Pivovarova *et al.* 1994b). The importance of the apical V-type ATPase was emphasised by the rise in $[K^+]_i$ when NEM was applied to the tubule (Pivovarova *et al.* 1994b). The possibility of movement of Na^+ and K^+ across the apical membrane via the action of separate alkali metal/H^+ antiporters was initially advanced by Weiczorek (Weiczorek 1992), based on investigations on GCAM from *Manduca sexta* midgut (Weiczorek *et al.* 1991). However, this area remains contentious, since the putative apical exchanger(s) remain uncharacterised; no proteins with homology to known vertebrate Na^+/H^+ exchangers have been identified (Dow *et al.* 1997). Furthermore, no clear consensus has been reached regarding whether the exchanger(s) are electroneutral or electrogenic. Weiczorek *et al.* (1991) favoured an electrogenic stoichiometry of at least $K^+/2H^+$ for *Manduca sexta* GCAM cation/proton antiport, in light of free enthalpy calculations, voltage profiles, and ISM measurements of K^+ and H^+ activities (Moffet & Koch 1988a,b; Chao *et al.* 1991; Weiczorek *et al.* 1989, 1991). Commonly, the antiporter has therefore been defined as K^+/nH^+, though the majority of previously identified K^+/H^+ antiporters are electroneutral (Weiczorek *et al.* 1991). In *Formica* Malpighian tubules, the apical K^+/H^+ exchanger is thought to be electroneutral with a one for one exchange of cations and protons (Leyssens *et al.* 1993; Van Kerkhove 1994; Zhang *et al.* 1994). At the apical surface, anion efflux was thought to be passive in *Locusta* Malpighian tubules (Baldrick *et al.* 1988), probably via Cl^- channels, which have been identified in the Malpighian tubules of *Aedes* (Wright and Beyenbach 1987) and proposed in *Drosophila hydei* (Bertram *et al.* 1991).

A heterodox stance was recently adopted to explain apical K^+ transport in the thread hair sensilla of *Periplanta americana*, where it was suggested that the efflux of K^+ into the receptor lymph cavity was effected directly by a K^+-V-ATPase (Küppers & Bunse 1996). Weiczorek and Harvey (1997) have argued against the presence of such a membrane protein, since it requires an unlikely mutation of an existing proton specific V-type ATPase, and because the parallel activity of a proton pumping V-type ATPase and cation/H^+ antiporter does not in fact violate the Second Law of Thermodynamics, as hypothesised (Küppers & Bunse 1996). The apical presence of a V-type ATPase was confirmed in *Locusta* Malpighian tubules on the basis of cross-reaction between an apical membrane fraction subunit and a monoclonal antibody raised to *Manduca sexta* midgut V-

type ATPase (Al fifi *et al.* 1998). The presence of a putative Na^+-K^+-$2Cl^-$ cotransporter has been identified in *Manduca* Malpighian tubules, with homology to vertebrate bumetanide-sensitive cotransporters, and it was speculated that phosphorylation of several sites might lead to the activation of this protein since a number of putative protein kinase C phosphorylation sites are thought to be present, as well as a protein kinase A site (Regan 1995). The action *Manduca* diuretic hormone was earlier suggested to involve the activation of the Na^+-K^+-$2Cl^-$ cotransporter (Audsley *et al.* 1993), possibly mediated by cAMP, as in shark rectal gland (Xu *et al.* 1994). The rate of fluid secretion is markedly accelerated in *Manduca* Malpighian tubule cells through the action of cAMP when diuretic hormone binds to its receptor (Regan 1994, 1995).

In *Rhodnius*, the earlier observations that 5-HT was stimulatory to fluid production were substantiated by the finding that this amine was present in biologically active amounts *in vivo* (Maddrell *et al.* 1991). The free haemolymph concentration of 5-HT was thought to be sufficient to at least stimulate partially fluid secretion in fed individuals of this species, via cAMP (Maddrell *et al.* 1991). Post-prandial stimulation of fluid production in *Rhodnius* was proposed to be maximal when diuretic hormone (in the form of mesothoracic ganglion extract) and 5-HT interacted synergistically, via the action of adenylate cyclase (Maddrell *et al.* 1993). The rate of fluid secretion was substantially increased in the presence of both 5-HT and DH, if compared with the effect of either of these diuretic hormones alone (Maddrell *et al.* 1993). In spite of an *in vivo* role for this biogenic amine, the majority of insect diuretic factors are neuropeptides (Coast 1996). These fall into 2 classes, namely CRF-related peptides, so-called because of their structural homology with the corticotropin-releasing factor/sauvagine/urotensin I family of vertebrate peptides, and the insect kinin family of neuropeptides (Coast 1996). Following the earlier finding by Mordue (1969, 1972) that neurosecretory cells of the pars intercerebralis synthesised diuretic hormone in *Locusta migratoria*, and transported this hormone to the storage lobes of the corpora cardiaca, attempts to identify the nature of this hormone were made (Morgan & Mordue 1985; Morgan *et al.* 1987). The diuretic activity of the extract was found to separate into two peaks (Morgan and Mordue 1983a), which suggested that the hormone existed in two (or more) forms. Kay *et al.* (1991) identified a CRF-related peptide, present in the pars intercerebralis and corpora cardiaca, which was shown to stimulate fluid secretion in isolated

Locusta Malpighian tubules and elevate intracellular cAMP levels (Coast *et al.* 1993). This was known as *Locusta*-DP; it was observed be present in the haemolymph of post-feeding locusts (Patel *et al.* 1995), and was released by a calcium-dependant mechanism from corpora cardiaca glands *in vitro* when incubated in a depolarising K^+-rich saline (Patel *et al.* 1994). *Locusta*-DP stimulated fluid secretion and raised cAMP to a similar extent as corpora cardiaca extract, and was considered to be an *in vivo* diuretic hormone in *Locusta migratoria* (Patel *et al.* 1995).

Members of the insect kinin family have been identified in numerous species of insect, including *Leucophaea maderae* (Holman *et al.* 1986a,b; Holman *et al.* 1987a,b) and *Locusta migratoria* (Schoofs *et al.* 1992). Initially identified in the former species, they are potent myotropic agents in isolated hindgut and have diuretic activity in *Locusta* (Coast *et al.* 1993; Coast 1996). *In vivo* application of locustakinin I, the locust kinin, stimulated fluid secretion and accelerated the rate of amaranth clearance, strongly suggesting an hormonal function for this peptide (Coast 1996). Where locustakinin differed from *Locusta*-DP is that it had no stimulatory effect on the intracellular level of cAMP (Thompson *et al.* 1995). Locustakinin I, like the *Leucophaea* kinin leucokinin VIII (Hayes *et al.* 1989), was isolated from brain-corpora cardiaca-corpora allata-suboesophageal ganglion complexes (Schoofs *et al.* 1992). The stimulatory effect on tubule fluid production of achetakinin, the kinin family peptide identified in *Acheta domesticus* was also considered to be cAMP-independent (Coast *et al.* 1990), but instead was responsible for a rise in the $[Ca^{2+}]_i$, possibly activating protein kinase C (Coast *et al.* 1993). Several leucokinins were initially identified from *Leucophaea*, and a synthetic form of one, leucokinin VIII (LK 8), was applied to *in vitro Aedes aegypti* Malpighian tubule cell preparations (Pannabecker *et al.* 1993; Wang *et al.* 1996). LK 8 was seen to increase the rates of NaCl, KCl and water secretion across the tubules, and the paracellular conductance of Cl^- (Pannabecker *et al.* 1993). It was thus suggested that 2 independent pathways existed in *Aedes*, the cAMP-regulated Na^+-dependent transcellular path, and a paracellular septate junctional path activated by LK 8 to increase Cl^- (Wang *et al.* 1996). Both were able to upregulate secretion of fluid, but only LK 8 could increase paracellular transport (Wang *et al.* 1996). A similar Cl^- shunt is present in *Drosophila melanogaster*, but this though to occur transcellularly via the second cell type in this species, the stellate cell (O' Donnell

et al. 1996). It has been shown that Cl^- conductance is stimulated by leucokinin 1 in *Drosophila*, probably by a rise in $[Ca^{2+}]_i$ (O' Donnell *et al.* 1996). Building on earlier suggestions by Morgan and Mordue (Morgan & Mordue 1984b) in *Locusta*, synergism between *Locusta*-DP and locustakinin in stimulating the rate of fluid secretion was recorded (Coast 1995,1996). Clearly the actions of the two peptides were mediated by separate second messengers since *Locusta*-DP could be replaced by 8-bromo-cAMP, and locustakinin by thapsigargin, to elicit a similar response (Coast 1995). In addition to the second messengers cAMP and Ca^{2+}, *Drosophila* Malpighian tubules cells have been demonstrated to secrete fluid at an increased rate as a probable consequence of nitric oxide stimulated catalysis of GTP to form cGMP, stimulating the apical V-type ATPase indirectly through protein kinase G (Davis *et al.* 1995; Dow *et al.* 1994; Davis *et al.* 1997). The likelihood of cross-talk between the NO-cGMP and Ca^{2+} signalling pathways in this tissue adds further complexity to the control of fluid production in this species (Davis *et al.* 1997).

Objectives.

The preceding section is summarised in a schematic diagram showing the current model of ion flux across *Locusta* Malpighian tubules (see Figure 1.10). This model proposes the basolateral surface is characterised by the presence of a classical Na^+-K^+-ATPase, an electroneutral Na^+-K^+-$2Cl^-$ cotransporter and by a basolateral conductance for K^+ leading to its efflux down a concentration gradient. The basolateral surface is considered to be energised in the conventional manner (Weiczorek & Harvey 1997), that is via P-type ATPase-mediated active efflux of Na^+ leading to the development of a driving force for K^+ re-entry through the cotransporter. The apical membrane is considered to be energised by a proton-motive V-type ATPase (Weiczorek & Harvey 1997), facilitating the movement of both alkali metal cations into the tubule lumen by the action of separate cation/nH^+ antiporters (Al-fifi *et al.* 1998a). Luminal Cl^- efflux is thought passive down a favourable electrochemical gradient. A transcellular transport route, through the type 1 fluid secreting cell could conceivably account for all the Cl^- movement in this epithelium, as a consequence of the basolateral presence of the Na^+-K^+-$2Cl^-$ cotransporter. There is no evidence either for or against the existence of an alternative Cl^- shunt, as is

found in both *Aedes aegypti* and *Drosophila melanogaster* Malpighian tubule (O' Donnell *et al.* 1996; Wang *et al.* 1996). In this way, *in vivo* transport of K^+ from the haemolymph to the tubule lumen is linked to the production of tubule fluid by solute-water coupling.

Nevertheless, this model is far from settled in many aspects. Doubt has been expressed regarding the exact contribution of the basolateral cotransporter to ion transport in *Locusta* tubule cells (Baldrick 1987; Baldrick *et al.* 1988; Fogg *et al.* 1993), and as noted earlier, the existence of "dedicated" Na^+ and K^+ antiporters remains to be proved. The status of paracellular routes for ion transport, particularly Cl^-, is unclear in *Locusta* (Fathpour *et al.* 1994) though such a mechanism has been suggested in *Aedes* (Wang *et al.* 1996).

Corpora cardiaca extract from *Locusta* stimulates intracellular levels of cAMP and IP_3 (Fogg *et al.* 1990). As noted, nervous tissue extract from this species has been shown to contain 2 diuretic factors, *Locusta*-DP and locustakinin, whose stimulatory actions on fluid secretion are mediated by cAMP and Ca^{2+} respectively (Coast 1996). In *Aedes*, binding of a CRF-related peptide is responsible for the stimulation of a basolateral Na^+-K^+-$2Cl^-$ cotransporter through the second messenger cAMP (Hegarty *et al.* 1991); similarly, binding of the CRF-related peptide Mas-DH occurs to a specific receptor in *Manduca sexta* midgut, which is coupled to the adenylate cyclase-cAMP second messenger system (Regan 1994). Identification of a putative-bumetanide sensitive Na^+-K^+-$2Cl^-$ cotransporter with possible sites for phosphorylation by PKC and PKA led to speculation that activation of this protein is effected by such phosphorylation. It is thus proposed that in *Locusta*, stimulation of fluid production by corpora cardiac extract is partially achieved by binding of *Locusta*-DP to an uncharacterised basolateral plasma membrane protein, coupled by adenylate cyclase and cAMP to the basolateral Na^+-K^+-$2Cl^-$ cotransporter, causing the phosphorylation of this protein through PKA and its subsequent activation. Further, it is tentatively suggested that locustakinin activates a second arm of the stimulatory process, again binding to an unidentified basolateral plasma membrane protein, and through the action of G protein/PLC, elicits a rise in $[Ca^{2+}]$ and an activation of PKC. The PKC/Ca^{2+} pathway may cause the activation of the basolateral Na^+-K^+-ATPase (Servala & Davy 1993; Chen *et al.* 1993), and it is tempting to speculate that co-operative stimulation by PKA and PKC phosphorylation cascades may be necessary for complete cotransporter

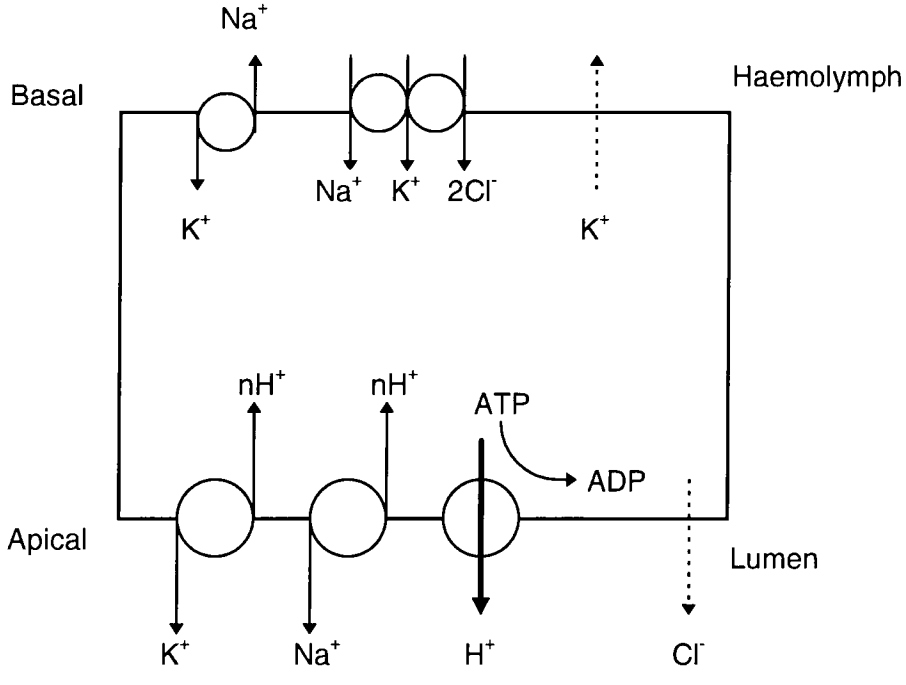


Figure 1.10. The current model for ion transport in *Locusta* Malpighian tubule cells.

activation. PKC has been observed to be an important stimulatory regulator of Na^+-K^+-Cl^- cotransport in NIH-3T3 fibroblasts (Hichami *et al.* 1996). The possibility of a locustakinin-stimulated paracellular flux of Cl^- may also be reasonably suggested (Wang *et al.* 1996). The observation that corpora cardiaca extract and exogenous stimulation with cAMP has a differential effect on both the cation composition of the secreted fluid, and the rate of the secreted fluid, further supports the reasoning that full stimulation of fluid production cannot be achieved by cAMP alone (Al-fifi *et al.* 1998a). Clearly, enhanced fluid secretion through peptide-induced changes in the levels of intracellular second messengers will evoke also changes in apical ion transport. It has been proposed that corpora cardiaca extract is responsible for an increase in apical anion conductance, and a differential change in the transport of Na^+ and K^+ across this membrane (Fogg *et al.* 1990; Al-fifi *et al.* 1998a). cAMP alone causes no change in the tubule fluid Na^+/K^+ ratio, and only increases fluid production by 50% of the value of corpora cardiac extract (Al-fifi *et al.* 1998a). It is interesting to note that *Locusta*-DP elicited a similar effect on the Na^+/K^+ ratio of the secreted fluid as corpora cardiaca extract, whilst locustakinin caused no change in the ratio (Coast 1995).

By combining measurements of secretion rates, cationic fluid content and fluxes from *in vitro Locusta migratoria* Malpighian tubule preparations, and intracellular elemental concentrations, the routes by which tubule cells convey ions from haemolymph to lumen have been investigated. This approach follows that of Pivovarova *et al.* (1993, 1994a,b) and Wessing and co-workers (Wessing *et al.* 1988, 1992, 1993, 1997; Wessing & Zierold 1990, 1992, 1993, 1996). By perturbing tubule functioning with stimulators and inhibitors of fluid production, it is hoped to elucidate further information regarding ionic movement and its control in this typical secretory epithelia. Specifically, basolateral plasma membrane events have been studied using the loop diuretic inhibitor furosemide, specific for Na^+-K^+-Cl^-, and the stimulators dibutyryl cAMP and corpora cardiaca extract; the possible stimulatory effect of a synthetic peptide locustatachykinin II (Schoofs *et al.* 1990) was investigated, as a possible locustakinin analogue. The apical membrane was expressly targeted by inhibition of secretion with bafilomycin A_1, and confirmation was sought for earlier findings into the effect of the potassium mimic rubidium on ion transport (Pivovarova *et al.* 1994a,b).

Intracellular measurements have also been recorded from cytoplasmic mass dense concretions in the presence of different stimulators; these concretions are a feature of Malpighian tubules in a number of species (Maddrell 1977; Wessing *et al.* 1992; Pivovarova *et al.* 1994a). These structures are somewhat enigmatic, but may be involved in the storage of cations such as K^+, acting as a labile pool within the ionic economy of the cell (Pivovarova *et al.* 1994a; Wessing & Zierold 1996). Thus, chapter three will describe the effect of the above-mentioned stimulators and inhibitors on the rate of fluid secretion, the concentration of Na^+ and K^+ in the secreted fluid and their associated calculated cationic fluxes. Chapter four details the effect of the same compounds on intracellular elemental concentrations and the content of the cytoplasmic mass dense concretions. A general discussion comprises chapter five, and is followed by two short appendices, the first describing the basis of biological X-ray microanalysis. This introduction is followed by a brief description of the general methods and materials used in this study.

Chapter Two.

Methods and Materials.

2.1. Insect maintenance.

A population of *Locusta migratoria* L. phase gregaria were maintained in an insectary at a temperature of 30±1°C, and a relative humidity of 50%. The animals were kept on a light/dark cycle of 12L:12D and maintained at a population density sufficient to prevent reversion to the solitary phase (Joly & Joly 1953). Between 50 and 70 locusts were kept in each of a number of glass fronted cages, of dimensions 41x41x60cms (Philip Harris Biological Ltd., Oldmixon, Western-Super-Mare, Avon). Each cage was fitted with a single 25W light bulb. Within each cage, humidity and temperature varied with distance from the light bulbs and time since fresh grass was introduced; relative humidity was not less than 60% and the temperature approximately 30-36°C. The insects were fed on fresh grass every day. Each generation took 40 to 50 days from emergence to reach the fifth instar and a further 10 to 14 days to reach sexual maturity.

2.2 Materials.

Plastic containers and glassware were washed throughout in detergent and rinsed six times in tap water, and twice in distilled water, before being allowed to dry either in a drying oven or in the air. For atomic absorption spectrophotometry, glassware was subsequently thoroughly rinsed in 30% (v/v) nitric acid, before further rinsing in distilled, MilliQ-grade water, and oven drying.

All reagents used were AnalaR grade or the best commercially available. Bafilomycin A_1, dibutyryl cyclic AMP (N^6, 2'-0-Dibutyryladenosine 3':5'-cyclic monophosphate, henceforth DB-cAMP) and furosemide (5-[Aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)amino]-benzoic acid) were all purchased from Sigma Aldrich Co. Ltd., Poole, Dorset. Locustatachykinin II (Lom-TK II) was purchased from Peninsula Laboratories Europe Ltd., St. Helens, Merseyside.

2.3 Insect physiological saline solutions.

Table 2.1 shows the composition of the physiological saline solutions used in this study. In all cases MilliQ-grade distilled water was used to make up the saline. Each solution was brought to pH 7.2 at room temperature using 1M NaOH solution (Fogg, 1990; Pivovarova *et al.* 1994a,b). In experimental solutions which required DMSO as a additional solvent, final DMSO concentration was 0.1%.

Table 2.1. The composition of saline solutions used in this study.

Reagent	Control saline (mM)	Rb saline (mM)
NaCl	100.0	100.0
KCl	8.6	-
$MgCl_2.\ 6H_2O$	8.5	8.5
NaH_2PO_4	2.0	2.0
$NaHCO_3$	4.0	4.0
NaOH	4.0	4.0
Glucose	11.0	11.0
HEPES	34.0	34.0
$CaCl_2$	25.0	25.0
RbCl	-	8.6

2.4 Electron microscopy.

All electron microscopy was carried out using a Philips 400T transmission electron microscope, the gun voltage being 100kV. The following description refers exclusively to the preparation, fixation, embedding and staining of tissue for conventional TEM. A detailed discussion of the preparation of tubule cells for X-ray microanalysis will be found in chapter four, and appendix one.

2.4.1 Preparation and fixation.

Malpighian tubules were obtained from sexually mature adults of either sex. The animal's abdomen was severed close to its posterior tip, anterior to the

8th or 9th abdominal tergite. The animal was decapitated by twisting the head, breaking the neck membrane and swiftly pulling upwards to extract the attached alimentary canal through the pronotum. The Malpighian tubules were quickly dissected free whilst bathed in control saline, then fixed for 30 minutes to 1 hour in Karnovsky's fixative (Karnovsky 1965). This fixative has two components: (a). 2g paraformaldhyde in 40 ml dH_2O, dissolved by heating to 60°C plus a few drops of 1M NaOH and (b). 50 ml 0.2M sodium cacodylate buffer plus 10 ml 25% gluteraldhyde. (a) and (b) were mixed immediately prior to use. The tubules were subsequently post-fixed for 1 hour in 1% buffered osmium tetroxide (buffered in 0.2M sodium cacodylate).

2.4.2 Dehydration.

Once the tubules had been briefly washed in 0.1M sodium cacodylate, they were progressively dehydrated, firstly with 3 washes in 70% EtOH, each wash lasting 5 minutes. This was repeated in 95% EtOH. Finally, in 100% EtOH, each of the 3 washes lasted 10 minutes.

2.4.3 Infiltration and embedding.

The tubules were then infiltrated with a 1:1 100% EtOH/propylene oxide mix, then in propylene oxide alone (3 washes of 10 minutes in each case). During this time, the embedding resin was prepared, see table 2.2 below;

Table 2.2. The composition of Araldite embedding medium.

Reagent	**Volume (ml)**
Araldite CY212	10
dodecenyl succinic anhydride (DDSA)	10
n-Benzyldimethylamine (BDMA)	0.4
Dibutyl phthalate	1.0

The first three reagents were thoroughly mixed and warmed to 45°C before adding dibutyl phthalate immediately prior to use.

Next the tubules were infiltrated in 1:1 propylene oxide/embedding medium mix for 30 minutes at 45°C, and then for a further 30 minutes in pure embedding medium. Finally, the tubules were oriented in a suitable mould, covered with fresh embedding medium and polymerised at 45°C for approximately 1½ to 2 days.

2.4.4 Sectioning.

Following polymerisation, sections were cut using an RMC MT 6000 ultramicrotome. Glass knives with a cutting angle of 4° were used to cut sections of between 40 and 100 nm thickness (gold to purple coloured sections). The sections were stretched using chloroform vapour before mounting on square mesh copper grids.

2.4.5 Staining.

Sections were then stained sequentially for 10 minutes in each of uranyl acetate (1% in 10% EtOH) and lead citrate to resolve fine ultrastructural detail (Reynolds 1963).

2.5 Statistical analysis.

Statistical analyses were routinely performed using the spreadsheet MS Windows Excel version 5.0. The significance of the difference between two sample means was tested using either a paired t-test or Student's t-test (Sokal & Rohlf 1981). Both equal and unequal variance t-tests were carried out, the results of F_{MAX}-tests for variance being used to determine the most appropriate t-test. Kolmogorov-Smirnov tests of normality were performed in the statistical package SPSS for Windows. Mann-Whitney U-tests for non-parametric data were carried out in the statistical package Minitab version 11. Data is presented throughout the study as mean ± standard error of the mean. In all cases, a probability of less than 0.05 was regarded as statistically significant.

Chapter Three.

The rate and composition of the fluid secreted by *in vitro* preparations of *Locusta migratoria* L. Malpighian tubules.

3.1. Introduction.

In vitro measurement of the rate of fluid production by insect Malpighian tubules has proved a fundamental experimental tool for studies on secretory epithelial physiology. This experimental technique is both simple and flexible. It is possible to investigate both the rate of fluid production and the composition of the fluid produced by isolating a number of tubules in an artificial "haemolymph", the experimental saline solution. The method used here is that previously described by Anstee and Bell (1975), which was a slightly modified version of that of Maddrell and Klunsuwan (1973). This technique allows the tubules to remain undisturbed and well oxygenated once the experiment has begun, and the system can also be maintained at a constant, physiologically appropriate temperature (Anstee & Bell 1975; Bell 1977; Anstee & Bowler 1979).

Ramsay was the first to use *in vitro* preparations of insect Malpighian tubules and to measure the rate of production of tubule fluid (Ramsay 1954). Many later studies on Malpighian tubule cell secretion were based on this *in vitro* tubule preparation (Berridge 1966a,b, 1968; Maddrell 1962, 1963, 1964a,b, 1969; Rafaeli-Bernstein & Mordue 1978; Morgan & Mordue 1981; Dow *et al.* 1994). The ionic flux has also been studied with radiolabelled cations using a similar *in vitro* technique (Fogg *et al.* 1993). Morgan and Mordue have found *in vitro* preparations suitable as a bioassay for the role of cAMP (Morgan & Mordue 1985) and the identification of diuretically active fractions from corpora cardiaca gland extracts in *L. migratoria* (Morgan *et al.* 1987). More recently, variation of this basic tubule preparation has been developed to study extracellular pH gradients near the basolateral surface of *Rhodnius* tubules (Collier & O'Donnell 1997).

The approach adopted in this and the next chapter follows that of Pivovarova *et al.* (1994a), where fluid secretion rates, fluid cation content and

intracellular elemental concentrations from *Locusta* Malpighian tubule cells were combined to provide a comprehensive picture of the *in vitro* functioning of the tubule cell. The data presented on the rate of fluid secretion and the concentration of Na^+ and K^+ in this fluid allows an understanding of the movement of ions across this secretory epithelia since solute-water coupling is considered to link the movement of K^+ and water into the lumen of *Locusta* tubules, thus forming the tubule fluid (Ramsay 1956).

The effect of the Na^+-K^+-ATPase inhibitor ouabain on the composition and rate of fluid secretion was examined using these experimental techniques in *Locusta*, confirming the presence of this enzyme in the tubule cells (Anstee *et al.* 1979). Further studies in our laboratory have used other inhibitors (Kalule-Sabiti 1985; Baldrick 1987) and stimulators of tubule fluid secretion (Fogg 1990). More recently, staurosporine and chelerythrine were demonstrated to inhibit fluid production, whilst dibutyryl cAMP and corpora cardiaca extract stimulated it (Al-fifi 1997; Al-fifi *et al.* 1998a). In all these cases, as in this study, the ultimate aim is to examine fluid production (and by inference ion transport) in the presence of compounds and cation mimics which alter this production, to comprehend better the processes which facilitate secretion.

Figure 3.1 shows the currently favoured model of ionic transport in *Locusta* Malpighian tubule cells. Details of this model are given in chapter one, but briefly, the model requires a basolateral Mg^{2+}-dependant Na^+-K^+-ATPase (Anstee & Bell 1975), and a loop diuretic-sensitive cotransporter with a stoichiometry of $1Na^+$:$1K^+$:$2Cl^-$ (Maddrell & O'Donnell 1984). Some doubt has been cast upon the presence of this cotransporter in *Locusta* (Baldrick 1987; Baldrick *et al.* 1988; Fogg *et al.* 1993). Basolateral K^+ efflux is thought to be passive down its concentration gradient. Secondary active transport of K^+ across the tubule cell apical surface via a K^+/nH^+ antiporter is considered to be energised by a proton pumping V-type ATPase (Bertram *et al.* 1991). A similar dedicated Na^+-transporting antiporter may be present, (Pivovarova *et al.* 1994a) though the possibility of a common antiporter for both K^+ and Na^+ has not been excluded (Al-fifi *et al.* 1998a). A large favourable electrical gradient enables passive efflux of chloride across the apical plasma membrane (Baldrick *et al.* 1988).

In this chapter, the response of *Locusta* Malpighian tubule cells to a range of stimulators and inhibitors of fluid secretion has been examined. The

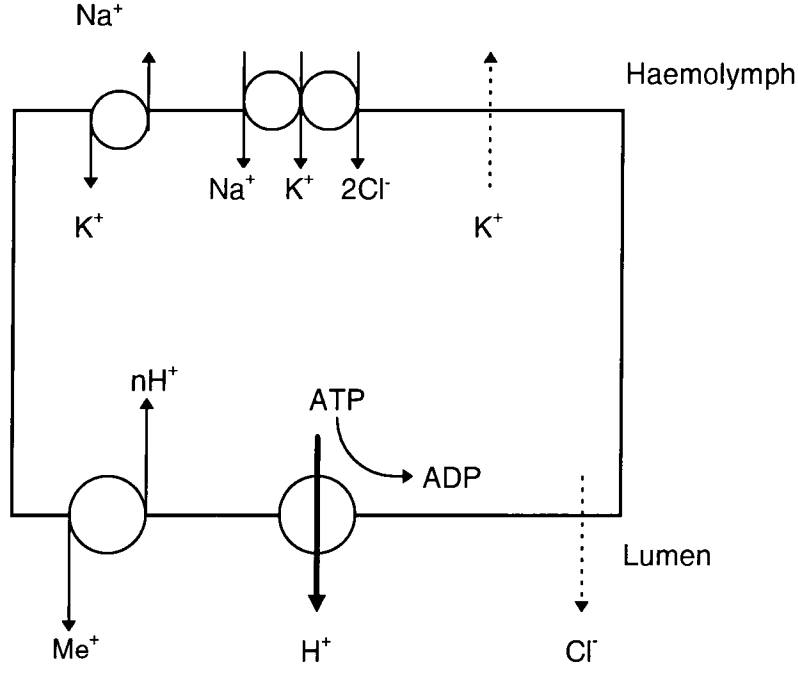


Figure 3.1. General model of ion transport in *Locusta* Malpighian tubule cells. Me^+ represents either Na^+ or K^+. (For symbols, see chapter one).

stimulators were 1mM diubutyryl cAMP, corpora cardiaca extract and Lom TK II. cAMP and corpora cardiaca both accelerate fluid production in *in vitro* preparations of *Locusta* Malpighian tubules (Al-fifi *et al.* 1998a), but addition of exogenous cAMP alone did not elicit as great a stimulatory response as corpora cardiaca extract (Morgan & Mordue 1984b; Al-fifi *et al.* 1998a). Diuretic hormone from the corpora cardiaca raises $[cAMP]_i$, and also increases intracellular levels of IP_3 (Fogg *et al.* 1990). Data on the rate of fluid secretion data obtained in the presence of these stimulants will be used in conjunction with intracellular element concentrations obtained by X-ray microanalysis (see chapter 4) to test the suggestion that these second messengers modulate the activity of transport proteins.

LomTK II (Locustatachykinin II) is a myotropic decapeptide isolated from the brain - corpora cardiaca - corpora allata suboesophageal ganglion complex of *L. migratoria* (Schoofs *et al.* 1990). A synthetic analogue of this peptide causes spontaneous hindgut contractions in the cockroach *Leucophaea maderae* (Schoofs *et al.* 1990). Lom TK II shares this ability with a family of octapeptides, the leucokinins. These have been isolated from *L. maderae*, and one, leucokinin VIII (LK 8), has been shown to stimulate Malpighian tubule fluid production in *Aedes aegypti* (Hayes *et al.* 1989). LK 8 depolarises the TEP in *Aedes*, which is dependant upon the presence of chloride in the bathing saline (Hayes *et al.* 1989). This is reminiscent of the effect of corpora cardiaca extract upon *Locusta* tubules (Fogg *et al.* 1989). Insect kinins have no effect upon $[cAMP]_i$ in *Acheta* since their activity is mediated by the Ca^{2+} second messenger pathway (Coast *et al.* 1993). On the basis of common myotropic activity between locustatachykinins and leucokinins, it is suggested here that Lom TK II may stimulate fluid production, possibly mediated by a rise in $[Ca^{2+}]_i$ alone.

The inhibitors used were 1mM furosemide and 1µM bafilomycin A_1. Furosemide inhibits Na^+-K^+-Cl^- cotransport; it has been previously shown to inhibit fluid production in *Locusta* Malpighian tubules (Kalule-Sabiti 1985; Baldrick 1987; Baldrick *et al.* 1988). The composition of the tubule fluid and rate of secretion resulting from inhibition by this loop diuretic will be examined. The effect of the specific and highly potent inhibitor of V-type ATPases, bafilomycin A_1 (Dröse & Altendorf 1997), on both the rate and composition of the secreted fluid has been investigated with the intention of confirming the importance of this ATPase in ionic transport across this epithelia.

An equimolar concentration of rubidium was also used as a mimic for potassium, particularly as K^+ is considered to be the prime mover for tubule fluid production in these cells (Ramsay 1956). Confirmation has been sought for the inability of Rb^+ to maintain optimal fluid secretion rates, in the absence of K^+ (Pivovarova *et al.* 1994a). The effect of 1mM furosemide in the same Rb-containing saline was investigated. In common with the Na^+-K^+-ATPase in fibroblasts (Longo *et al.* 1991), the Na^+-K^+-Cl^- cotransporter replaces K^+ with Rb^+ efficiently in avian erythrocytes (Palfrey *et al.* 1980), Erlich ascite tumour cells (Geck *et al.* 1980) and human fibroblasts (Longo *et al.* 1991). By using Rb^+-containing saline and 1 mM furosemide in conjunction, information has been gained regarding the contribution of Na^+-K^+-Cl^- cotransport to unstimulated ion transport in *Locusta* Malpighian tubules.

3.2 Methods.

3.2.1 Measurement of the rate of Malpighian tubule cell fluid production.

The rate of tubule fluid production by *Locusta* tubules was measured as follows. The abdomen of sexually mature adult locusts of either sex were severed close to the posterior end of the animal, the individual was then decapitated and the whole of the alimentary canal removed through the neck. The Malpighian tubules, which remained attached to the alimentary canal at the midgut, were quickly rinsed in control physiological saline (at 30±1°C), and the whole of the alimentary canal placed in a specially designed dish (see Figure 3.2) (Maddrell & Klunsuwan 1973).

The shallow depression in which the alimentary canal rested contained physiological saline; the whole dish was then covered with a layer of paraffin oil. The dish itself was placed within a brass chamber, connected to a water bath maintained at 30±1°C. The saline solution and the oil surrounding it was periodically checked to ensure the temperature remained constant.

Initially during a 20 minute equilibration period, up to 10 tubules were draped around the metal pins in the dish, and punctured using a fine tungsten needle (Maddrell & Klunsuwan 1973). As a result, small, roughly spherical droplets of tubule fluid were seen to be produced by the punctured tubules. The rate of fluid production was recorded by measuring the increase in diameter of the droplets of

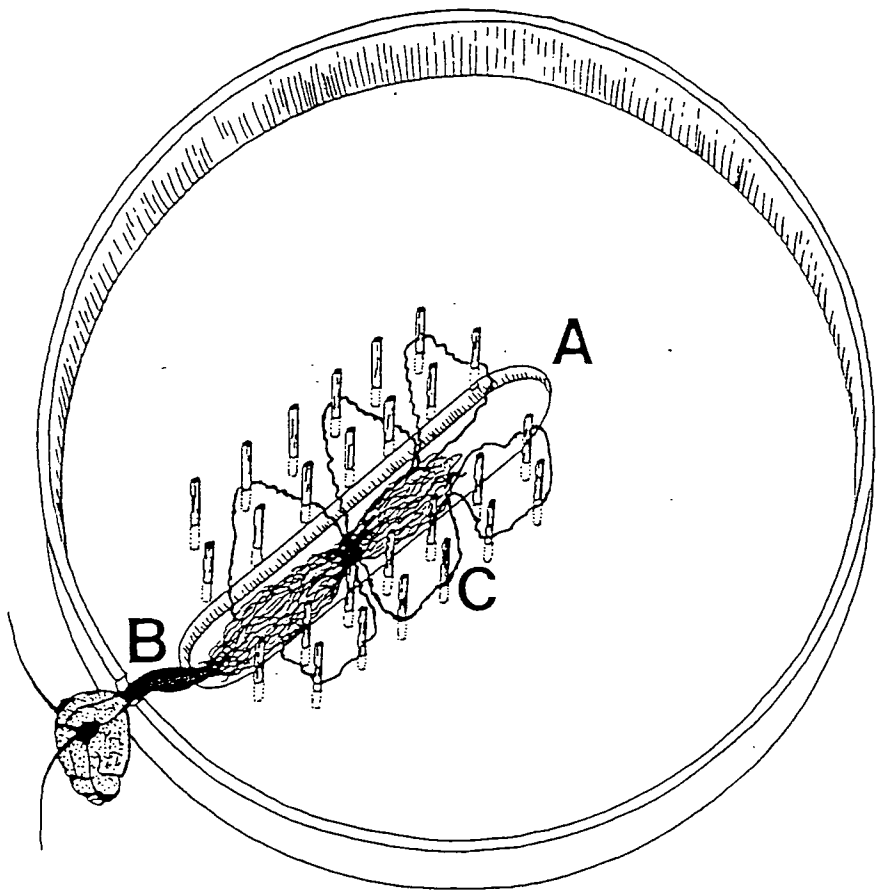


Figure 3.2. *In vitro* Malpighian tubule preparation for rate of fluid secretion and tubule fluid collection experiments. An insect's alimentary canal (with the head attached, protruding from the dish) is seen in position, immediately prior to tubule puncturing. The central depression (A) contains physiological saline, and the insect's head is separated from the saline by a small piece of Blue-Tak (not shown), at B. The dish is covered with liquid paraffin and placed in an water-filled heating jacket, connected to a 30±1°C water bath. Malpighian tubules are seen (labelled C) looped around metal pins, and would be surrounded by paraffin oil. For clarity, only 6 tubules are shown, though 10 or more may be drawn out from a single animal. (Diagram adapted from Bell,1977).

fluid, with an eyepiece graticule attached to a suitably positioned dissecting microscope. Assuming an approximately spherical droplet, the volume was calculated from the diameter of the droplet.

After the 20 minute equilibration period, the droplet diameters were recorded every 5 minutes for 30 minutes; this was referred to as **Rate 1** secretion. Subsequently, the saline was replaced with the appropriate experimental saline and the tubules left to equilibrate for a further 20 minutes before the rate of secretion was again measured over 30 minutes. This was known as **Rate 2** secretion. The diameters of the droplets were then converted to volumes and the rates (in nl min^{-1}) were determined graphically.

3.2.2. Analysis of urine composition.

The collection of secreted fluid followed the same general pattern as the measurement of the rate of secretion. Adult female locusts were dissected and prepared according to the method detailed in section 3.2.1 (above). Female animals were used in these experiments since it has been suggested that they secret a larger volume of fluid during R2 than tubules from males (Al-fifi, pers.comm.). This was considered critical as several of the experiments in this chapter used fluid production inhibitors which substantially slowed the rate and total volume of secreted fluid. The tubules were punctured with clean glass microelectrodes in order to eliminate ionic contamination. Fluid droplets were allowed to develop under the paraffin oil for 45 minutes before they were pooled together. The pooled droplets were then collected in a 1µl capillary tube and diluted in 4ml of distilled, MilliQ-grade water prior to atomic absorption spectrophotometry. As before, the control saline was then replaced with an appropriate experimental saline and the urine pooled and collected after 45 minutes. Therefore, urine was collected during two 45 minute periods equivalent to Rates 1 and 2, both equilibration periods being the same as detailed in section 3.2.1.

Atomic spectrophotometry was performed using a Perkin-Elmer 5000 atomic absorption spectrophotometer. $[Na^+]$ and $[K^+]$ were measured on absorption at 589nm and 766.5nm respectively. Calibration solutions of KOH and NaOH (BDH, ConVol) were prepared, and diluted to an appropriate concentration before analysis. Figures 3.3 and 3.4 show the calibration curves for Na^+ and K^+ respectively.

Figure 3.3. Standard curve for [Na+]

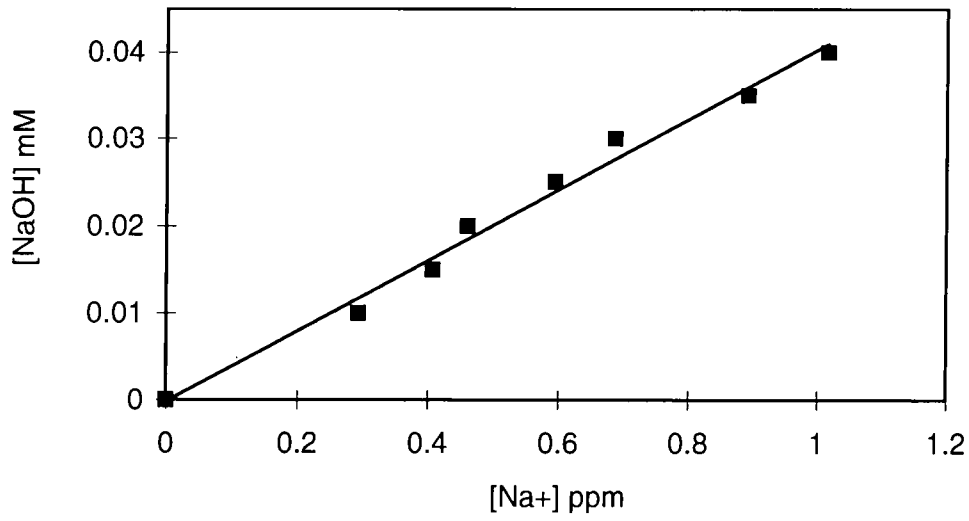
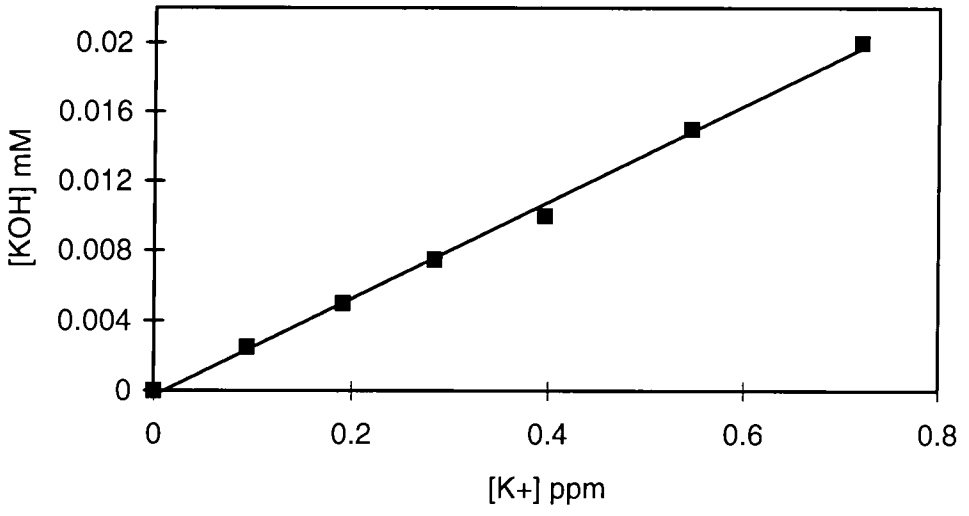


Figure 3.4. Standard curve for [K+]



3.2.3. Preparation of corpora cardiaca extract.

To an prepare extract, ten gland pairs were dissected from adult male insects under ice-cold control physiological saline, added to 0.1 ml fresh ice-cold control saline and homogenised using a Teflon coated pestle, in a glass homogeniser surrounded by ice (clearance 0.10 - 0.15 mm); approximately 20 passes at 1000 revs/min were required. The resulting extract was diluted to 10

ml of saline, and kept ice-cold until shortly before use, when it was rapidly warmed to 30°C. Fresh extract was prepared each day.

3.3.Results.

Rate of fluid production.

3.3.1. Control experiment.

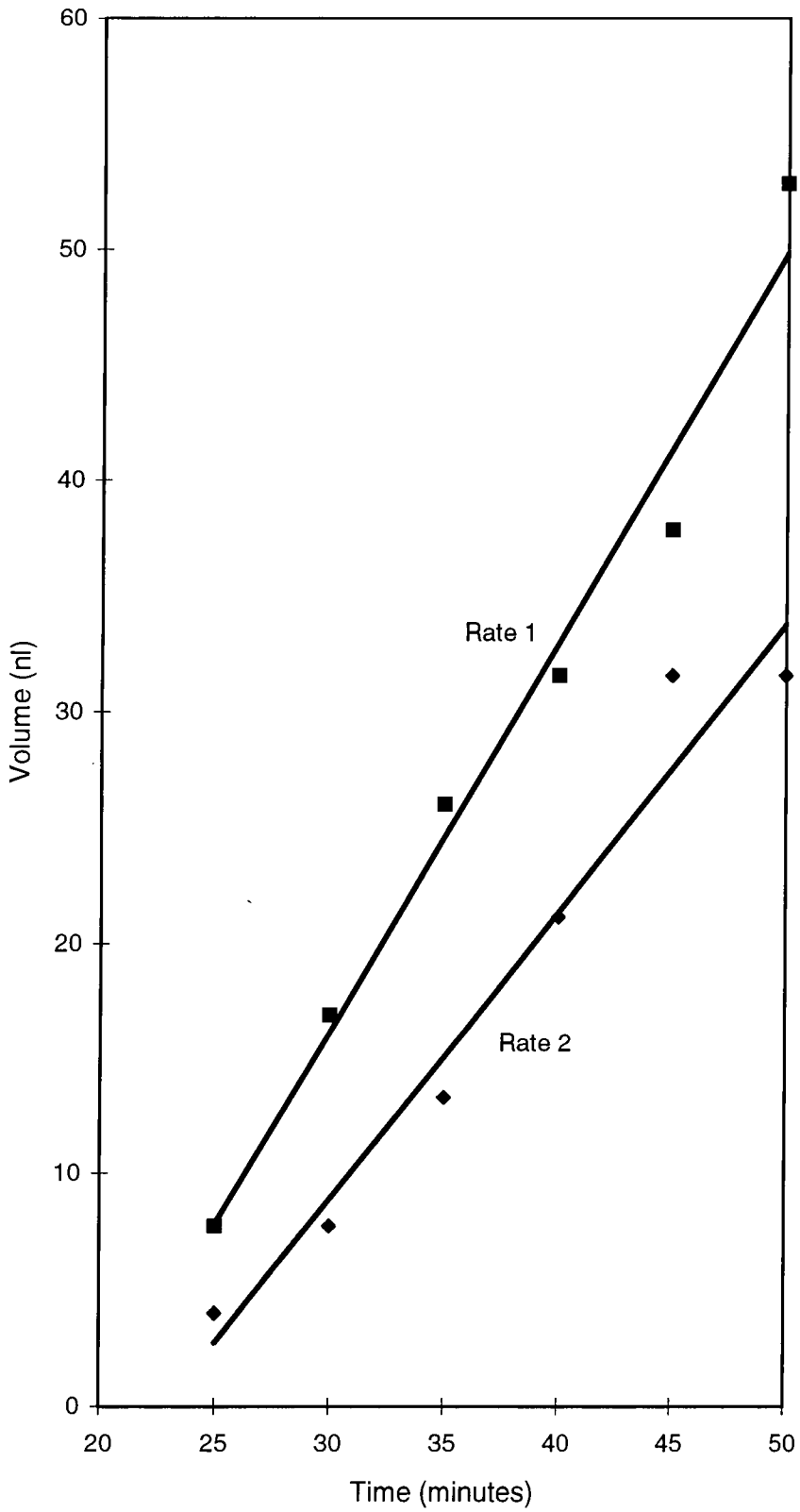
The following data were collected from animals incubated in control saline solution for both the Rate 1 and Rate 2 experimental periods. Throughout the rate of secretion data presented in this section, each tubule acted as its own control, that is, during every treatment Rate 1 secretion was measured when the tubules were bathed in control saline alone. Only during the Rate 2 period were saline solutions containing the appropriate inhibitor or stimulator of fluid production added to the *in vitro* tubule preparation.

Table 3.1. Rates of fluid secretion and R2/R1 % values for control tubules.

Rate 1 (nl min^{-1})	Rate 2 (nl min^{-1})	R2/R1 %	n
1.820±0.127	1.333±0.121	84.41±6.17	78

A paired t-test indicated that R2 was significantly lower than R1 ($t=4.15$, $P<<0.05$). The R2/R1% value in Table 3.1 and all the subsequent tables in this chapter does not represent the percentage calculated directly from the Rate 1 and Rate 2 data columns. Instead, it is a mean value itself, derived from the R2/R1% of each tubule. This is calculated for each tubule from that tubule's R1 and R2 values, and is the mean normalised rate of secretion for each tubule, where rate 1 equals 100%. Figure 3.5 shows a typical graph of the rate of secretion of tubule fluid from a Malpighian tubule *in vitro*. The tubule was from a female locust; Rate 1 secretion was calculated as 1.795 nl min^{-1}, Rate 2 was 1.475 nl min^{-1} and R2/R1 was 82.17%. The secretion rate varied considerably between individual tubules. Some tubules failed to secrete when punctured. Such tubules were discarded. To test the normality of distribution of the two

Figure 3.5. The control rates of fluid secretion from a typical Malpighian tubule *in vitro*. Squares represent rate 1 secretion, diamonds, rate 2.



experimental data sets, a series of Kolmogorov-Smirnov (KS) tests were performed. The results of these tests are presented in appendix two, and led to the conclusion that the control data were normally distributed. Therefore all the subsequent rate of secretion data in this chapter was analysed assuming that it is normally distributed.

3.3.2. Effects of inhibitors on fluid production.

Table 3.2 illustrates the rates of fluid production and the R2/R1% values measured from *in vitro* preparations of *Locusta* Malpighian tubule cells, incubated in the fluid production inhibitor indicated. As noted in section 3.3.1, in all the treatments listed in Table 3.2, Rate 1 (R1) refers to the 30 minute period when the fluid secretion rate was measured as the tubules were incubated in control saline alone. During Rate 2 (R2), the tubules were incubated in the appropriate fluid secretion inhibitor, again for 30 minutes. Thus, the title of the treatment in the saline column of Table 3.2 refers to the composition of the saline during the Rate 2 period.

Table 3.2. Rates of fluid secretion and R2/R1% values of *in vitro* preparations of *Locusta* Malpighian tubules incubated in fluid production inhibitors.

Saline	**Rate 1** ($nl\ min^{-1}$)	**Rate 2** ($nl\ min^{-1}$)	**R2/R1** %	**n**
Control	1.820±0.127	1.333±0.121	84.41±6.17	78
0.1% DMSO	1.919±0.208	1.271±0.175	70.58±5.99	29
1mM furosemide	1.419±0.131	0.511±0.096	38.21±6.22	23
1µM bafilomycin	2.274±0.505	0.262±0.095	15.27±5.55	12
Rb saline	2.061±0.261	0.753±0.138	39.72±6.40	33
Rb saline plus 1mM furosemide	2.097±0.277	0.681±0.136	33.40±7.33	19

The effect of 1mM furosemide on the rateof fluid production.

Examination of the data in table 3.2 demonstrates both controls, i.e. control and 0.1% DMSO saline, had similar R1 ($t=0.26$, $P>0.05$), R2 ($t=0.22$, $P>0.05$) and R2/R1% ($t=1.61$, $P>0.05$). The R2/R1% of the tubules incubated in 1mM furosemide saline was significantly lower than that of the control ($t=5.28$, $P<0.001$), as was its R2 rate ($t=5.32$, $P<0.001$). However, a comparison of the control and furosemide R1 data indicates that these also differed significantly ($t=2.203$, $P<0.05$). Paired t-tests indicate that both 0.1% DMSO and 1 mM furosemide R1 and R2 are significantly different ($t=4.49$, $P<0.001$ and $t=6.77$, $P<0.001$ respectively).

Nevertheless, since the R2/R1% value of the 1mM furosemide was lower than that of the control, and this was not the case for the 0.1% DMSO saline, it can be concluded that furosemide significantly decreased the rate of fluid production in the Malpighian tubules. This result clearly highlights the importance of using each tubule as its own control. Though the R1 secretion rate was lower in the furosemide experiment than the control (and during this period the saline solutions are the same for both the control and the furosemide experiments), a comparison of the R2/R1% values of these data sets shows there was a significant decrease in the rate of fluid production on addition of furosemide saline.

The effect of 1µM Bafilomycin A_1 on the rate of fluid production.

As may be readily seen from Table 3.2, 1µM bafilomycin A_1 had a dramatic and highly significant inhibitory effect upon the rate of fluid secretion. The difference between the control and bafilomycin A_1 R2/R1% values was highly significant ($t=8.34$, $P<0.001$). Furthermore, the control and bafilomycin A_1 R1 results were not significantly different ($t=0.87$, $P>0.05$), whilst the bafilomycin R2 value was significantly smaller than the control ($t=6.98$, $P<0.001$). A paired t-test shows that the 1 µM bafilomycin R2 rate is significantly lower than R1 ($t=3.98$, $P<0.05$).

The effect of replacing K with Rb on the rate of fluid production.

Student's t-tests confirm that there was no significant difference between the control and Rb saline R1 rate of secretion ($t=0.83$, $P>0.05$). The R2 and

R2/R1% values were very highly significantly different, however ($t=3.16$, $P<0.05$ and $t=5.03$, $P<0.001$ respectively). It may be concluded that replacing K with Rb in the incubation saline leads to a decrease in the rate of fluid production. The R2/R1% of Rb-saline treated tubules is not significantly different from that of furosemide treated tubules ($t=0.164$, $P>0.05$). A paired t-test shows that the Rb-saline R2 is significantly less than the R1 ($t=5.58$, $P<0.001$).

The effect of 1mM furosemide on the rate of fluid production in Rb-saline.

The Rb plus 1mM furosemide R1 did not differ from either the control or the Rb saline R1 ($t=0.95$, $P>>0.05$ and $t=0.09$, $P>>>0.05$ respectively). Rb plus furosemide R2 and R2/R1% results were significantly lower than the control values ($t=3.58$, $P<<<0.05$ and $t=5.16$, $P<<<0.05$ respectively). In comparison with the Rb saline experiment, both the Rb plus furosemide R2 and R2/R1 % were not statistically significantly different ($t=0.35$, $P>>>0.05$ and $t=0.61$, $P>>0.05$ respectively). As with tubules incubated in Rb-saline only, a paired t-test indicates that R2 value is significantly less than the R1 in tubules incubated in Rb-saline plus furosemide ($t=5.86$, $P<0.001$). Furosemide therefore appears to elicit no additional inhibition of fluid production, above the level already seen as a result of the replacement of K by Rb.

3.3.3. Stimulators of fluid production

Table 3.3 gives the rates and R2/R1% values from tubules incubated in stimulators of fluid production. Only during rate 2 are the *in vitro* tubule preparations incubated in saline containing the stimulator. The control values presented in Table 3.3 are taken from the control data set (see table 3.1) but use data recorded from female preparations only. Tubules from male insects were not stimulated to secrete at a faster rate by either DB-cAMP or corpora cardiaca extract.

Table 3.3. Rates of fluid secretion and R2/R1% values of *in vitro* preparations of *Locusta* Malpighian tubules incubated in fluid production stimulators.

Saline	Rate 1 (nl min^{-1})	Rate 2 (nl min^{-1})	R2/R1 %	n
Control	1.973±0.246	1.630±0.269	88.96±10.75	31
1mM DB-cAMP	2.737±0.732	3.921±0.997	149.75±18.15	9
Corpora cardiaca extract	2.097±0.246	3.407±0.392	174.79±22.96	13
10µM Lom TK II	2.314±0.333	1.852±0.333	87.87±11.97	16

The effect of 1mM DB-cAMP on the rate of fluid production.

Table 3.3. shows the result of the addition of 1mM dibutyryl cyclic AMP (DB-cAMP) to the bathing saline on the rate of fluid secretion by *in vitro* preparations of female Malpighian tubules. A paired t-test comparing the control tubules' rate 1 and rate 2 secretion rates indicated these were not significantly different ($t=1.731$, $P>0.05$).

By comparing the secretion rates during the Rate 1 period of the control and control plus cAMP-treated tubules, it is clear that there was no significant difference between these two rates ($t=0.990$, $P>0.05$). Similarly, during Rate 2, when the cells are stimulated by the addition of cAMP, the secretion rate was not significantly different from the control rate ($t=2.219$, $P>0.05$). Nevertheless, an increase in the rate of secretion was reflected in the enhanced R2/R1% value of the cAMP treated cells ($t=2.733$, $P<0.05$). A paired t-test showed there was no significant difference between the R1 and R2 secretion rates of cAMP treated tubules ($t=1.441$, $P>0.05$).

The effect of corpora cardiaca extract on the rate of fluid production.

Table 3.3. reveals the effect of the addition of 0.1 corpora cardiaca gland pairs per ml of control saline, on the rate of fluid secretion of female tubules. The tubules were incubated in control saline plus corpora cardiaca extract during the R2 period.

There was no significant difference between the rates of secretion of the control and the experimental tubules during the Rate 1 period ($t=0.303$, $P>0.05$). Clearly, the addition of corpora cardiaca gland extract substantially accelerates the secretion rate; Rate 2 secretion was more than three times greater in tubules exposed in corpora cardiaca extract than control tubules ($t=3.648$, $P<0.001$). Similarly, the R2/R1% of the corpora cardiaca extract was very much greater than the control ($t=3.866$, $P<0.001$). A paired t-test reveals the corpora cardiaca rate 2 secretion rate was significantly greater than the rate 1 ($t=3.965$, $P<0.05$).

The effect of 10μM Lom TK II on the rate of fluid production.

Table 3.3. shows the effect of the addition of 10μM Lom TK II on the rate of fluid production. The Rate 1 secretion rates were not significantly different ($t=1.556$, $P>0.05$). Neither the Rate 2 secretion rates ($t=1.697$, $P>0.05$) nor the R2/R1% values ($t=0.236$, $P>>0.05$) differed significantly between the control and Lom TK II treated cells. Amongst the Lom TK II treated tubules a paired t-test demonstrated that there was no significant difference between the rate 1 and rate 2 rates of secretion ($t=1.908$, $P>0.05$). Clearly LomTK II did not increase the rate of tubule fluid production.

Cationic composition of the secreted fluid.

3.3.4. Control experiments.

K^+ and Na^+ concentration and flux in fluid secreted by Malpighian tubules incubated in control saline.

The cationic concentration in the secreted fluid was measured using atomic absorption spectrophotometry. The first rate period served as a control, and during the second period, saline containing the appropriate fluid secretion inhibitors was applied to the tubules. Estimated cationic fluxes have been derived from the product of concentration of the cation in the tubule fluid and the volume of fluid collected.

Table 3.4 gives the K^+ and Na^+ concentrations and cation fluxes in the secreted fluid when the tubules are incubated in control saline. $[Na^+]$ and $[K^+]$

data were tested for normality of distribution using Kolmogorov-Smirnov test, and was found not to deviate from the normal distribution. The results of these tests may be found in appendix two. In consequence, the remainder of the data in this section have been analysed assuming it is normally distributed.

Table 3.4. $[K^+]$, $[Na^+]$ and cation flux in secreted fluid from tubules incubated in control saline.

Rate period	$[K^+]$ mM	K^+ flux (nmoles min^{-1})	$[Na^+]$ mM	Na^+ flux (nmoles min^{-1})	n
Rate 1	126.08± 3.49	1.970± 0.251	50.79± 5.11	0.743± 0.077	9
Rate 2	128.74± 6.50	1.295± 0.155	51.16± 4.98	0.485± 0.046	9

Paired t-test showed that there was no significant difference between the $[K^+]$ ($t=0.848$, $P>0.05$) and $[Na^+]$ ($t=0.047$, $P>0.05$) during the Rate 1 and Rate 2 periods. The mean sum of the two cations is 176.88 ± 5.98 mM during R1 and 179.90 ± 7.52 mM during R2. The $[K^+]$: $[Na^+]$ ratio is approximately 2.5 in for both periods. The flux of cations, derived as the product of volume and the cation concentration of tubule fluid collected for analysis is also shown. Analysis using paired t-tests indicated that the K^+ and Na^+ fluxes during the R1 and R2 periods were significantly different from each other ($t=2.40$, $P<0.05$ and $t=3.12$, $P<0.05$). The K^+/Na^+ flux ratio during Rate 1 was 2.63 ± 0.21 and during Rate 2 was 2.75 ± 0.32; a paired t-test indicated these values were not significantly different ($P=0.298$, $P>0.05$).

K^+ and Na^+ concentration in fluid secreted by Malpighian tubules incubated in control saline plus 0.1% DMSO.

Table 3.5 shows the effect of control saline plus 0.1% DMSO, when added to the bathing saline during Rate 2 secretion.

Table 3.5. [K^+] and [Na^+] in secreted fluid from tubules incubated in control saline plus 0.1% DMSO.

Rate period	[K^+] mM	[Na^+] mM	n
Rate 1	130.10± 7.10	53.01± 7.56	8
Rate 2	131.16± 4.91	47.78± 5.89	8

There was no significant difference between the [K^+] and [Na^+] of fluid secreted by tubules in control saline during Rate 1 and in control saline plus 0.1 % DMSO during Rate 2 as analysed by paired t-tests (t=0.212, P>0.05 and t=0.503. P>0.05 respectively). The [K^+] during both Rate 1 and Rate 2 (as detailed in table 3.5 above) was not significantly different from the equivalent rate period K^+ concentration in the control experiment (table 3.3.9.1) (t=0.122, P>0.05 and t=0.291, P>0.05, respectively). Neither did the Na^+ concentrations differ if the control and control plus DMSO experiments rate 1 and rate 2 concentrations were compared (t=0.545, P>0.05 and t=0.291 P>0.05). These results show that 0.1% DMSO has no effect upon the K^+ and Na^+ concentrations in the secreted tubule fluid.

3.3.5. Inhibitors of fluid production.

K^+ and Na^+ concentration and flux in fluid secreted by Malpighian tubules incubated in control saline plus 1mM furosemide (in 0.1% DMSO).

Table 3.6 illustrates the effect of 1 mM furosemide on composition of the secreted fluid, and the cation flux, when the saline plus 1 mM furosemide is added during the Rate 2 period.

Table 3.6. $[K^+]$ and $[Na^+]$ and cation flux in secreted fluid from tubules incubated in control saline plus 1mM furosemide (in 0.1% DMSO).

Rate period	$[K^+]$ mM	K^+ flux (nmoles nl^{-1} min^{-1})	$[Na^+]$ mM	Na^+ flux (nmoles nl^{-1} min^{-1})	n
Rate 1	127.46± 4.68	2.052± 0.189	50.91± 4.60	0.804± 0.081	13
Rate 2	107.21± 7.21	0.611± 0.410	77.42± 8.76	0.410± 0.048	13

1mM furosemide had a significant effect on the Na^+ concentration of the secreted fluid. The $[Na^+]$ in the secreted fluid collected after the Rate 2 period was significantly greater than that seen in the fluid produced during the control saline-only Rate 1 period, as a paired t-test indicated ($t=2.751$, $P<0.05$). An additional paired t-test revealed the difference in the $[K^+]$ during the rate 2 period was not statistically significant ($t=1.953$, $P>0.05$). When the $[K^+]$ and $[Na^+]$ concentrations during the rate 1 secretion period were compared with the equivalent concentrations during the control experiment described in Table 3.4, no significant difference was observed ($t=0.217$, $P>0.05$ and $t=0.0174$, $P>0.05$ respectively). The $[K^+]$:$[Na^+]$ ratio decreased from approximately 2.5 to 1.4 in response to incubation with furosemide. The cation flux data displayed a decrease in the flux ratio (i.e. flux of K^+ / flux of Na^+) from 2.70 ± 0.21 during Rate 1, to 1.69 ± 0.19 during Rate 2. A paired t-test confirmed this decrease was statistically significant ($t=3.106$, $P<0.05$).

K^+ and Na^+ concentration and flux in fluid secreted by Malpighian tubules incubated in control saline plus 1µM bafilomycin A_1.

Table 3.7 indicates the change in fluid cationic flux and composition when 1µM bafilomycin A_1 was incorporated in the incubation saline during Rate 2 secretion period.

Table 3.7. $[K^+]$ and $[Na^+]$ and cation flux in secreted fluid from tubules incubated in control saline plus 1 µM bafilomycin A_1.

Rate period	$[K^+]$ mM	K^+ flux (nmoles nl^{-1} min^{-1})	$[Na^+]$ mM	Na^+ flux (nmoles nl^{-1} min^{-1})	n
Rate 1	140.07±13.09	1.790± 0.379	55.54± 5.66	0.673± 0.135	9
Rate 2	97.94±16.32	0.550± 0.130	97.63±16.21	0.554± 0.156	9

Paired t-tests showed 1 µM bafilomycin A_1 reduced the $[K^+]$ in the fluid produced by Malpighian tubules ($t=2.763$, $P<0.05$) and induced a significant rise in the Na^+ concentration ($t=3.245$, $P<0.05$). With regard to both $[Na^+]$ and $[K^+]$, the values reported in the fluid during the Rate 1 period in Table 3.7 were not significantly different from those in the control experiment (Table 3.4) (t 0.623 $P>>0.05$ and $t=1.033$ $P>>0.05$). The $[K^+]$: $[Na^+]$ ratio decreases from approximately 2.5 to 1.0. The flux ratio of K^+/Na^+ was once more lowered in the presence of an inhibitor. The flux ratio during Rate 1 was 2.70 ± 0.28, and during Rate 2 was 1.19 ± 0.27. The decrease observed was significantly different according to a paired t-test ($t=6.40$, $P<<0.05$). The difference between the Na^+ fluxes during Rate 1and Rate 2 was significant, as analysed by a paired t-test ($t=2.49$, $P<0.05$).

3.4. Discussion.

Control and control saline plus 0.1% DMSO.

A wide variation in the control rate of tubule fluid secretion by individual Malpighian tubules was observed in this study. In the control data set presented in this investigation, secretion rates varied from a maximum of 6.154 nl min^{-1} to a minimum of 0.389 nl min^{-1}. Earlier work with *Locusta* also demonstrated substantial differences in this secretion rate (Bell 1977; Kalule-Sabiti 1985; Baldrick 1987). This feature of secretion by *in vitro* tubule preparations has been observed in other species, for example *Aedes aegypti* (Williams & Beyenbach 1983; Hegarty *et al.* 1991; Beyenbach *et al.* 1993). The reasons for this variation may include the differences in the age and nutritional state of the insect, and the

dimensions of the individual punctured tubule. The protozoan parasite *Melamoeba locusta* is occasionally present in large numbers intraluminally in adult *Locusta migratoria*, and can be seen in electron micrographs causing the tubule cells to be compressed against the basement membrane (author's unpublished observation). Proux has indicated that infection in this species renders individuals unable to respond to diuretic factor, though the action of the intracellular second messenger cAMP seems to be unaffected (Proux 1991). In consequence, insects with visible necrotised tubules were excluded from *in vitro* experiments, though the parasites may be present in the lumen without the tubule displaying external damage (author's unpublished observation). The inherently variable nature of *in vitro* fluid production has been stressed in *Aedes*, where it was proposed to be a result of changes in the number and activity of plasma membrane transport proteins, arising from differences in the diet of individual insects (Beyenbach *et al.* 1993).

The mean values of secretion rates during the R1 and R2 periods (Table 3.1) are in agreement with previous workers' findings in *Locusta migratoria* Malpighian tubules. Bell (1977) reported a control rate of 1.7 nl min^{-1}, and Fogg (1990) 1.1 ± 0.2 nl min^{-1}. Similarly, Morgan and Mordue (1981) and Kalule-Sabiti (1985) gave their unstimulated control rates as 2-4 nl min^{-1}, the latter author suggesting a total range of rates of between 1 and 7 nl min^{-1}. Marshall (1995) recorded control secretion rate values of approximately 2.21 to 2.04 nl min^{-1} (Marshall 1995). Recent values quoted by Al-fifi in this species were approximately 4 to 4.5 nl min^{-1} (Al-fifi 1997; Al-fifi *et al.* 1998a). The R2/R1% value shown in Table 3.1 is similar to those reported by Bell (1977) of 98.3 ± 14.0 % in *Locusta*, also that of Marshall (1995) of 91.96 ± 1.96 %, and finally that calculated from the data of Al-fifi (1997), of 93.5%. In summary, the control secretion rate results from this study are generally in accordance with previous investigations.

However, in contrast to a recent study (Al-fifi 1997), during the control experiment described in section 3.3.1, the Rate 1 and Rate 2 rates of secretion were significantly different from each other if both male and female insects were included, as analysed by paired t-test. It is not clear why this might be so, but in section 3.3.3, a different picture emerges. When comparing secretion rates by females insects alone, no difference was noted between rates 1 and 2, as

observed earlier (Al-fifi 1997). These finding suggest that male insects may secrete tubule fluid more slowly than females during the rate 2 period.

Tubule fluid was collected for cation analysis from female insects only. Preliminary investigations suggested that female tubules produced a greater amount of fluid during the R2 period. This was considered to be an important advantage in the experiments carried out in this section as the total amount of fluid available for analysis was invariably less than 1 µl, and it was clear that this amount would be reduced in the presence of the inhibitors. The cation concentration data shown in Table 3.4 is in agreement with that presented by Al-fifi and co-workers (Al-fifi 1997; Al-fifi *et al.* 1998a), and with earlier work on *Locusta* tubules (Anstee *et al.* 1979; Pivovarova *et al.* 1994a). In this study, the reported value of $[Na^+]$ in the tubule fluid was more variable than $[K^+]$, with values in the range approximately 38 to 80 mM. Greater consensus was achieved in the values of the potassium concentration, the range being from 110 to 142 mM. The sum of the two cations was also similar to the results of Anstee *et al.* (1979). The ratio of $[K^+]$: $[Na^+]$ does not differ between the 2 control experiment collection periods and is close to recently obtained values (Al-fifi *et al.* 1998a).

Ramsay first showed that the $[K^+]$ of the tubule fluid of *Locusta migratoria* was greater than the $[Na^+]$ (Ramsay 1953). The recorded concentrations varied, but $[K^+]$ was never less than the $[Na^+]$. The average concentration of the former cation was 6.2 times greater than the $[K^+]$ found in the haemolymph. Haemolymph $[Na^+]$ was 75-100 mM and $[K^+]$ was 15-20 mM (Ramsay 1953). *Locusta* Malpighian tubules are able to excrete considerable amounts of K^+ *in vitro* even when the $[K^+]$ in the bathing saline is low (Anstee *et al.* 1979), and this is a feature of many other species' tubules e.g. *Calliphora* (Berridge 1968) and *Schistocerca* (Maddrell & Klunsuwan 1973).

These data confirm that K^+ is the predominant cation in the tubule fluid, and that active K^+ transport must occur during *in vitro* tubule fluid secretion, as the control saline solution $[K^+]$ is only 8.6 mM but the concentration of this cation in the tubule fluid is approximately 130mM. On the other hand, from the data presented here, Na^+ movement to the tubule lumen could be entirely passive since the saline $[Na^+]$ is 108 mM.

Inhibitors of fluid production.

Furosemide.

The data presented in Table 3.2 clearly indicates that 1mM furosemide substantially decreased the rate of fluid production in *Locusta* Malpighian tubule cells. Furosemide is a sulphurmoylbenezoic acid derivative, and is used clinically as a diuretic (Palfery *et al.* 1980). It, along with a related compound, bumetanide, is a member of a class of inhibitors known as loop diuretics, which inhibit Na^+ Cl^- reabsorbtion in the vertebrate thick ascending limb of the loop of Henlé, promoting diuresis (Greger *et al.* 1985).

The inhibitory action of furosemide on fluid production by insect Malpighian tubules has been reported previously in both *Locusta* (Kalule-Sabiti 1985; Baldrick 1987; Baldrick *et al.* 1988) and *Rhodnius* (O'Donnell & Maddrell 1984; Maddrell & O'Donnell 1992). The site of action of furosemide was first identified as being electrically neutral cotransport in Erlich ascite tumour cells by Geck and his co-workers (Geck *et al.* 1980). A stoichiometry of $1Na^+$:$1K^+$:$2Cl^-$ commonly characterises this form of ionic transport (Geck *et al.* 1980) though other ratios have been reported (Haas 1994). Such furosemide-inhibitable cotransport is also associated with cell volume regulation in avian erythrocytes (Palfery *et al.* 1980) and is present in a wide range of epithelial and non-epithelial cells (O'Grady *et al.* 1987). When 1mM furosemide was applied to *Rhodnius* Malpighian tubules, the TEP depolarised dramatically whilst V_a hyperpolarised; from the small depolarisation of V_b, it was inferred that the inhibitor's action was upon an electrically silent transport system and that Cl^- crossed the basal membrane via cotransport with Na^+ and K^+ (O'Donnell & Maddrell 1984). The change observed in the TEP was similar to that induced by Cl^- free bathing media (O'Donnell & Maddrell 1984). In *Locusta* tubules, 1mM furosemide reduced $^{36}Cl^-$ flux by approximately 50% (Fogg *et al.* 1993). Baldrick (1987) indicated that in a similar experiment to the one described in section 3.3.1, 1mM furosemide caused the R2/R1 value to decrease to 22.63 ± 3.04%. Kalule-Sabiti (1985) reported a value of 37.9 ± 4.46 % for 10^{-3} M furosemide, but found that below 10^{-4} M, furosemide had no significant effect on fluid secretion.

The data presented also suggests that fluid secretion is reliant, at least on furosemide-inhibitable Na^+-K^+-$2Cl^-$ cotransport, since the R2/R1 value decreased to 38.21±6.22%. This value is in close agreement with that of Kalule-Sabiti (1985) but is less than the inhibition reported by Baldrick (1987).

Furosemide, however, has also been shown to inhibit other mechanisms of ionic transport at concentrations similar to that used here, for example carbonic anhydrase, the classical Na^+-K^+ ATPase, anion self-exchange in vertebrate cells (Palfrey *et al.* 1980; Palfrey and Greengard 1981; Greger 1985), and Na-Cl and K-Cl cotransport (Haas 1994). Baldrick *et al.* (1988) reported that 1mM furosemide initiated a slow hyperpolarisation of V_b and V_a in addition to a gradual depolarisation of the TEP across *Locusta* Malpighian tubules. However, bumetanide had no effect upon V_b at a concentration of either 10^{-4} or 10^{-5} M, though both concentrations elicited a hyperpolarisation of V_a and the TEP (Baldrick 1987). Bumetanide may be without effect on V_b because in the unstimulated cell, the Na^+-K^+-Cl^- cotransport does not contribute to V_b. Since the cotransporter is electroneutral, this is to be expected. Furosemide's hyperpolarising effect on V_b may be a result of inhibition of other transport proteins (Haas 1994). The observed hyperpolarisation of V_b in response to furosemide may cause an influx of K^+ ions through the hypothesised basolateral anomalous rectifier K^+ channel (D. Hyde, personal communication). This might account for the finding that the tubule fluid [K^+] remains unchanged in the presence of furosemide, as will be now discussed.

The Na^+ concentration in the secreted fluid increased by approximately 27 mM when the cells were incubated in the presence of 1 mM furosemide. In contrast, no change occurred in the K^+. Since the secretion rate was substantially slowed during inhibition with furosemide , we may predict the flux of K^+ may be reduced. The derived data in Table 3.6 showed that indeed the fluxes of both cations decreased; additionally, Fogg and colleagues (Fogg *et al.* 1993) have demonstrated that ^{36}Cl flux is also substantially slowed in response to 1 mM furosemide.

The ability of furosemide to affect differentially cation concentration in the fluid suggests their transport is differentially regulated. The position of K^+ as the "prime mover" in fluid production is once again demonstrated, as the decrease in this cation's flux corresponded to a fall in the rate of tubule fluid production. Though the flux of K^+ decreased, the [K^+] in the tubule fluid remained unchanged. This suggests that the Malpighian tubule cells are able to compensate to some extent for inhibition at the basolateral surface. An alternative K store, besides the intracellular K^+ pool, may be available in these cells (Pivovarova *et al.* 1994a).

Bafilomycin A_1.

The data presented in Table 3.2 confirms that bafilomycin A_1 substantially reduces the rate of fluid secretion *in vitro*. This inhibitor is the most efficacious used in this study, used at a concentration of 1µM. It has been previously demonstrated (Marshall 1995) that bafilomycin A_1 reduces the secretion rate by *Locusta* tubules with a concentration of 5 µM giving an R2/R1 value of 27.3 ± 7.1%. A concentration of 0.5 µM elicited an R2/R1 value of 58.6 ± 5.8 % (Marshall 1995). In this study, of the twelve tubules treated with this inhibitor, only five achieved an R2/R1 % greater than 10%, the mean value being 15.27 ± 5.55%. In an *in vitro* preparation of larval *Drosophila hydei* tubules, fluid production was reduced at bafilomycin A_1 concentrations above 0.1 µM (Bertram *et al.* 1991). 1 µM decreased the rate to between 40 and 50 % of the control rate (Bertram *et al.* 1991).

Bafilomycin A_1 is a member of the macrolide family of antibiotics, and was first isolated from *Streptomyces griseus* (Dröse & Altendorf 1997). A total of four bafilomycins have been identified, namely A_1, B_1, C_1 and D (Bowman *et al.* 1988). Bowman *et al.* were the first to investigate systematically the inhibitory effect of bafilomycin on ATPases (Bowman *et al.* 1988). They observed that vacuolar ATPases from *N. crassa*, *Z. mays* and bovine adrenal medulla were highly sensitive to inhibition by bafilomycin A_1 (Bowman *et al.* 1988). Concentrations below 10^{-3} µmol per mg tissue resulted in such inhibition, but at higher concentrations of bafilomycin A_1 (approximately 4 µmol per mg tissue), other ATPases such as the classical Na^+-K^+ ATPase were affected (Bowman *et al.* 1988).

The sensitivity of insect secretory epithelia to bafilomycin A_1 was identified by Bertram and Weiczorek and their colleagues (Bertram *et al.* 1991; Weiczorek *et al.* 1991; Bertram *et al.* 1993). In view of the extreme specificity of this inhibitor for V-type ATPases when used at concentrations less than 0.5×10^{-3} µmol mg^{-1} (Bowman *et al.* 1988), Bertram *et al.* proposed that the reduction in fluid production was due to the presence of a V-type ATPase on the apical surface of the epithelial cell (Bertram *et al.* 1991). This followed the earlier discovery by Weiczorek and his co-workers that the apical membrane of tobacco hornworm (*Manduca sexta*) midgut possessed a V-type ATPase functionally coupled to a cation-H^+ antiporter (Schweikel *et al.* 1989; Weiczorek *et al.* 1989).

Later, in support of this, Weiczoreck's group showed that sub-micromolar concentrations of bafilomycin A_1 caused a large decrease in membrane bound ATPase activity in *M. sexta* purified goblet cell apical membranes (Weiczorek *et al.* 1991). In *Locusta* Malpighian tubules, the presence of an apical membrane V-type ATPase has been confirmed by immunocytochemical staining (Al-fifi *et al.* 1998b), as well as in *Formica polyctena* (Garayoa et al. 1995), *Heliothis virescens* (Pietrantonio & Gill 1995), *Manduca sexta* (Klein *et al.* 1991, Russell *et al.* 1992). Two further V-type ATPases inhibitors have been shown to limit fluid production in Malpighian tubules, n-ethyl maleimide (NEM) and 7-chloro-4-nitobenz-2-oxa-1,3-diazole (NBD-Cl), though they were 10 to 50 times less potent than bafilomycin (Bertram *et al.* 1991). In *Locusta*, fluid secretion was inhibited with NEM concentrations above 10^{-4} M (Marshall 1995), with 10^{-3} M NEM diminishing the R2/R1 % to 17.4 ± 5.0 %. Significant inhibition of fluid production (R2/R1 % = 70.8 ± 5.6 %) in *Locusta* with NEM at concentrations as low as 0.1 µM has been reported (Al-fifi 1997).

Bafilomycin has opposite and almost equal effects upon the $[Na^+]$ and $[K^+]$ in the tubule fluid. The K^+ concentration is lowered by approximately 43 mM and the Na^+ increased by a similar amount. The significant reduction in the $[K^+]$ may be explained by the inhibition of the V-type ATPase which is proposed to energise K^+ transport across the apical membrane (Bertram *et al.* 1991). The importance of this protein in tubule fluid production is clear from the dramatic reduction in the secretion rate and the parallel decrease in $[K^+]$ in the fluid which occurred in this study. Both treatment with furosemide and bafilomycin emphasises that in *Locusta* tubules, normal fluid secretion rates requires considerable K^+ movement into the tubule lumen, and also that Na^+ cannot effectively substitute for the former cation.

Rb-saline.

Rubidium is a member of Group I of the periodic table. When replacing 8.6mM KCl with 8.6mM RbCl in the bathing saline, the *in vitro* fluid secretion rate decreased so that the R2/R1% was a little under 40% (see Table 3.2). Rb-saline has therefore been classed as an inhibitor of fluid production. Similar results were reported earlier (Pivovarova *et al.* 1994a; Marshall 1995); the R2/R1% value was 50.70 ± 4.2 % in the latter of these two studies. Though the rate of secretion in Rb-saline declined considerably, $[Rb^+]$ remained low in the tubule

fluid (Pivovarova *et al.* 1994a). Berridge's investigations into *Calliphora* (Berridge 1968) showed that if the bathing saline surrounding an *in vitro* Malpighian tubule preparation was changed to include 140mM Rb in place of K, the rate of fluid production declined to from 13.8 $mm^3 \times 10^{-3}$ min^{-1} to 10.9 $mm^3 \times 10^{-3}$ min^{-1}. This represented a drop of approximately 21%.

Rb^+ has been used extensively as a marker for K^+ transport in physiological experiments (e.g. Shennan 1989; Wilson *et al.* 1990; Dörge & Rick 1990; Pivovarova *et al.* 1993, 1994a,b; Hichami *et al.* 1996), and in insect behavioural and feeding studies (for example Fernandes *et al.* 1997; O'Neill *et al.* 1997). The activity of the classical Mg^{2+}-stimulated Na^+-K^+-ATPase can be stimulated by monovalent cations other than K^+ in the following sequence $Rb^+ > NH_4^+ > Cs^+ > Li^+$ (Skou 1965), and in *Locusta* tubules the basolateral Na^+-K^+-ATPase can substitute Rb^+ for K^+ with 80% efficiency (Pivovarova *et al.* 1994a). The Na^+-K^+-$2Cl^-$ cotransporter's ability to utilise Rb^+ in this way is not known in insect tubules. It has been demonstrated in many other cell types that Rb^+ replaces K^+ at least as efficiently in Na^+-K^+-$2Cl^-$ cotransport as it does in the Na^+-K^+-ATPase; for example in human fibroblasts (Longo *et al.* 1991), Erlich ascite cells (Geck *et al.* 1980) or winter flounder intestine (O'Grady *et al.* 1986). K^+ channels in *Drosophila* (Perez-Cornejo & Begenisich 1994) are less permeable to Rb^+ than K^+, a result duplicated in mammalian exocrine acini (Gallacher *et al.* 1984). Also, K^+ channels in *Manduca sexta* midgut epithelia substitute Rb^+ for K^+ efficiently (Schirmanns & Zeiske 1991). Nevertheless, with the exception of two studies by Pivovarova and colleagues (Pivovarova *et al.* 1993, 1994a), relatively little is known of the tubule cell response to Rb^+ with regard to changes in V_a, V_b or the TEP, though after incubation for 45 minutes in saline containing 8.6mM RbCl, the tubule fluid $[Na^+]$ was unchanged from control values, but $[K^+]$ decreased (Pivovarova *et al.* 1994a). The secreted fluid contained approximately 12mM $[Rb^+]$ (Pivovarova *et al.* 1994a). Rb saline was unable to sustain the tubules' normal secretion rates. This is probably because the secretion of tubule fluid is dependant upon events at the apical as well as the basolateral surface. Solute-water coupling requires K^+ for exchange for protons via the apical K^+/nH^+ antiporter. Without plentiful K^+, the action of this antiporter will be inhibited, leading to reduced fluid secretion rates.

Rb-saline plus 1mM furosemide.

In vitro, the addition of furosemide to the Rb-bathing saline did not significantly decrease the rate of fluid production in comparison with Rb saline alone. These results suggest that furosemide does not additionally reduce the fluid secretion rate in Rb saline. Either Na^+-K^+-$2Cl^-$ cotransport plays no part in basolateral ionic transport in Rb^+ saline, or during unstimulated fluid production in Rb^+ saline electroneutral cotransport does not contribute to the net cation transport responsible for fluid production. Work conducted on amphibian skin epithelia principal cells favours the latter explanation, since it has been shown that Rb^+ uptake is negligible in cells inhibited with ouabain and amiloride (Dörge *et al.* 1989; Dörge & Rick 1990). Ussing has shown that in frog skin epithelia, loop diuretic sensitive cotransport is only activated during cell volume homeostasis (Ussing 1982). The conclusion that the cotransporter is "inactive", as defined above, is supported by a comparison between the R2/R1% values of tubules inhibited by 1mM furosemide and Rb saline alone. There is no apparent difference between these two treatments' secretion ratios.

1 mM DB-cAMP.

The R2/R1% value for female tubules incubated in DB-cAMP containing saline significantly increased from the control ratio of 88.96%, to 148.67%. However, a paired t-test indicated that the DB-cAMP rates 1 and 2 were not significantly different from each other. The evidence available is therefore somewhat equivocal regarding the stimulatory effect of cAMP. Comparison with the effect of Lom TK II on fluid production is instructive. In the latter case, neither the R2/R1% nor the Rate 2 and 1 secretion rates were significantly different from the control values; further, the Rate 2 and 1 secretion rates were not significantly different within this treatment. Stimulation with DB-cAMP however led to a raised R2/R1%, suggesting that at least partial stimulation of tubule fluid production was evoked by DB-cAMP. Nevertheless, cyclic nucleotide stimulation of insect secretory epithelia fluid production is well established. Malpighian tubules in *Rhodnius* and *Carausius* were stimulated to secrete tubule fluid at an accelerated rate by cAMP at concentrations of 1×10^{-4} and 1×10^{-5} M respectively (Maddrell *et al.* 1971). *Aedes aegypti* tubule fluid production increased on addition of DB-cAMP with a threshold of approximately 10^{-6} M, and with maximal stimulation at 10^{-4} M (Williams & Beyenbach 1984). In *Aedes*, DB-cAMP raised the mean secretion rate from 0.79 to 2.90 nl min^{-1} (Williams & Beyenbach 1984).

Berridge showed that the salivary glands of *Calliphora erythrocephala* responded to cAMP with a greatly increased secretion rate, within a range of 10^{-3} to 10^{-2} M (Berridge & Patel 1968). Even the Malpighian tubules of a desert species, the tenebrionid beetle *Onymacris plana* was greatly stimulated by 1 mM DB-cAMP, and could maintain a sustained secretion rate 4 to 8 times greater than the control rate for several hours (Nicolson & Isaacson 1986). For phytophagus species, *Schistocerca gregaria* quadrupled its secretion rate on stimulation with 5×10^{-5} M cAMP, from approximately 1 nl min^{-1} to over 4 nl min^{-1}(Maddrell & Klunsuwan 1973). In *Locusta*, Bell (1977) recorded an R2/R1 value of 273.1 ± 40.0% for 1mM cAMP stimulation, yet Al-fifi and co-workers (Al-fifi 1997; Al-fifi *et al.* 1998) observed a value of 150%. This value is close to that of Morgan and Mordue (1981), also in *Locusta*, who found that 5mM cAMP evoked a rise in the rate from approximately 4 to 6 nl min^{-1}. Other work in insects has established that cAMP causes tubule fluid production to increase (e.g. Petzel *et al.* 1987; Spring & Hazelton 1987; Cooper *et al.* 1988; Clark & Spring 1992).

The R2/R1% value recorded in this study is therefore consistent with the findings in *Locusta* of Morgan and Mordue (1981) and Al-fifi *et al.* (1998a), but is lower than that of Bell (1977). The larger increases seen in the rates from haematophagous species tubules, for example *Aedes* (Williams & Beyenbach 1984) can be attributed to the feeding behaviour of this species. Generally, adult unstimulated secretion rates are low, since substantial urine production only occurs periodically, in response to the homeostatic stresses imposed by blood feeding (and only in females) (Clements 1992). Stimulated tubule secretion rates in individuals of these species dwarf their control rates (e.g. Maddrell & O'Donnell 1992), since the animal must quickly rid itself of a considerable salt and plasma load from a blood meal which may be twice its own body weight (Stobbart 1977).

Raised levels of $[cAMP]_i$ in tubule cells in response to diuretic hormone was first discovered by Aston (1975) in *Rhodnius*. Diuretic hormone, extracted from the corpora cardiaca glands increased $[cAMP]_i$ in *Locusta* tubule cells (Morgan & Mordue 1985). Endogenous cAMP plays a role in the stimulation of fluid secretion *in vivo*, and it appears from this and other work that exogenous DB-cAMP activated the stimulated state of secretion, at least in part. The $[cAMP]_i$ will depend upon the balance between its synthesis via adenylate cyclase and degradation by phosphodiesterase (PDE). Methyl xanthines, notably IBMX, are PDE inhibitors and have been used to retain elevated levels of cAMP in *Locusta*

tubule cells, which has the effect of stimulating secretion (Morgan & Mordue 1985). IBMX has no effect on V_b but initiates a hyperpolarisation of both V_a and TEP, a finding replicated by the application of 1 mM DB-cAMP (Fogg *et al.* 1989). This may reflect a rise in apical K^+ efflux. Depolarisation of V_b in Cl^- -free saline under the stimulatory effect of cAMP (Fogg *et al.* 1989) suggests that anion transport across the basolateral surface is an integral part of the response to elevated *in vivo* $[cAMP]_i$. Recent data suggests that the full stimulated response of tubules to corpora cardiaca extract is different to that noted to exogenous cAMP alone (Williams & Beyenbach 1984; Nicolson & Isaacson 1987; Al-fifi *et al.* 1998a).

Corpora cardiaca extract.

The results of this study show that corpora cardiaca extract substantially stimulated the rate of fluid production by female tubules *in vitro*. Both the rate 2 and R2/R1% values were significantly greater than the control values. Importantly, a paired t-test showed the corpora cardiaca stimulated tubules mean rate 2 was significantly greater than its rate 1. Previous work has shown that corpora cardiaca extract stimulates the rate of fluid production to a rate 100% greater than the control value (Al-fifi 1997; Al-fifi *et al.* 1998a). This study confirms that finding with a control R2/R1% of approximately 89%, the C.C. extract R2/R1% is approximately 175%. Fogg (1990) reported Malpighian tubule secretion rates resulting in an estimated R2/R1% value which was somewhat lower, approximately 50% greater than the control. In *Locusta*, corpora cardiaca extract changes the cationic composition of the secreted fluid, with a rise in the concentration of Na^+ and a fall in the concentration of K^+ (Al-fifi 1997; Al-fifi *et al.* 1998a). Flux estimates suggested a much larger increase in the flux of Na^+ than K^+, in contrast to the effect of cAMP (Al-fifi *et al.* 1998a). Electrophysiological experiments on *Locusta* show that corpora cardiaca extract induces a gradual but significant hyperpolarisation of V_b, and a large and rapid depolarisation of V_a, with a resulting depolarisation of the TEP (Fogg *et al.* 1989; Fogg 1990). The hyperpolarisation of V_b will cause a basolateral influx of K^+ down an electrochemical gradient, and could also cause the opening of a putative K^+-specific inwardly rectifying channel (D. Hyde, pers. comm.). This response differs from that obtained by stimulation by cAMP or IBMX, in which a hyperpolarisation of V_a and the TEP occurs (Fogg *et al.* 1989). Nevertheless, the active factors in

corpora cardiaca extract have been shown to be peptides in both *Locusta migratoria* and *Acheta domesticus* (Morgan & Mordue 1983a; Coast 1989), and clearly involve the activation of the cAMP / adenylate cyclase pathway in *Locusta* (Rafaeli and Mordue 1982; Morgan and Mordue 1984b,c).

The data presented here supports preceding work, that indicates the extract from *Locusta* corpora cardiaca gland accelerates considerably the secretion rate of *in vitro* Malpighian tubule preparations. Evidence has accumulated that suggests as well as in *Locusta*, corpora cardiaca extract causes additional stimulation above that evoked by cAMP in other species. *Onymacris* tubules secrete at 20-30 nl min^{-1} faster when exposed to homogenate from the corpora cardiaca, rather than exogenous cAMP alone (Nicolson & Hanrahan 1986). Similarly, 8-bromo-cAMP only causes a stimulation of the secretion rate to 80% of that elicited by corpora cardiaca extract in *Acheta domesticus* (Coast & Kay 1994). As the stimulatory effect of cAMP on fluid secretion in this study was not statistically significant, no such comparison can be made here. Numerical comparisons between much data collected on the effect of diuretic hormone can be difficult, since the use of homogenates of neurosecretory glands and other parts of the nervous system often leads to differences, for example, in their *in vitro* concentrations.

Lom TK II.

Lom TK II caused no accelerating effect upon the rate of fluid secretion *in vitro* (see Table 3.3). This confirmed that both male and female insects' tubules were unaffected by 10µM Lom TK II. The following consideration of the effects of kinin and tachykinin-family peptides will be confined to Othoptera. Locustatachykinins were initially isolated from the brain- corpora cardiaca - suboesophageal ganglia complexes of locusts. Two myotropic peptides were identified with the following sequences (Schoofs *et al.* 1990a);

Lom TK I	GPSGFYGVR-NH_2
Lom TK II	APLSGFYGVR-NH_2

During the isolation and purification of these peptides, the bioassay measured the myotropic effect of various fractions on *Leucophaea maderae* hingut. Lom TK II was selected for testing since the synthetic analogue exhibited

a marginally lower concentration threshold for stimulation of hindgut contraction than Lom TK I (Schoofs *et al.* 1990a). Further work has shown the presence of an additional two Locustatachykinins, III and IV (Schoofs *et al.* 1991).

Like the locustatachykinins, leucokinins stimulate spontaneous contractions in the hindgut of *L. maderae* (Holman *et al.* 1986a,b; Holman *et al.* 1987a,b). The latter peptides, of which eight have been isolated and sequenced, are found in head tissue of this insect. In *Aedes*, the same peptides alter fluid secretion by the insects' Malpighian tubules. *In vitro*, LK 8 (see below) depolarises the TEP and stimulates tubule fluid production in a dose dependant manner.

Leucokinin 8 GADFYSWG-NH_2

It was expected that Lom TK II would stimulate fluid production in *Locusta* Malpighian tubules. This is clearly not the case, most likely due to differences in the C-terminal pentapeptide cores of the polypeptides. This region is essential to the biological activity of kinins in *Acheta* (Coast *et al.* 1990). In fact, work by Nässel and co-workers posits that Lom TK I is responsible for the *in vivo* release of adipokinetic hormone I from the corpora cardiaca of *L. migratoria* (Nässel *et al.* 1995). The exact mechanism of such release is unknown but may involve cAMP/adenylate cyclase. *Locusta* brain and neuronal tissue has an extensive array of different myotropic neuropepetides (Schoofs *et al.* 1993) and a myoinhibitory nonapeptide (Lom-MIP) has been found in the brain- corpora cardiaca -suboesophageal ganglia complex (Schoofs *et al.* 1996). Its presence in the glandular cells of the corpora cardiaca has led to speculation that it may be antagonistic to the release of AKH (Schoofs *et al.* 1996).

Chapter Four.

The intracellular elemental composition of *Locusta migratoria* L. Malpighian tubules.

4.1. Introduction.

Investigations in epithelial tissue which are confined to measurements of the rate and content of the secreted fluid provide limited information. In isolation, measurements of the rate of fluid secretion by Malpighian tubules allow only inference as to the nature of ionic transport across the tubule. These data are supplemented by analysis of the ionic composition of the tubule fluid, but even so, can only provide information regarding transtubular movement of cations and anions. Paracellular routes, for example, cannot be excluded. Since nothing is known of events within the cell itself, there is a limit to that which may be implied concerning the action of epithelial cell plasma membrane transport proteins. The results presented in the previous chapter provide useful information on the "output" of the tubule under controlled "input" conditions, but it is also desirable to analyse the intracellular events in *Locusta* Malpighian tubule cells under different secretory conditions.

A number of experimental techniques have been used to study such events in insect secretory epithelia. Ion-selective microelectrodes have been utilised to measure the concentration of K^+, Na^+ and Cl^- intracellularly, and in droplets of tubule fluid, as well as the pH of both the cell and lumen of epithelia tissue (Berridge & Schlue 1977; Gupta *et al.* 1978; Leyssens *et al.* 1992, 1993a,b, 1994; Wessing *et al.* 1993; Dijkstra *et al.* 1995; Zhang *et al.* 1994; Collier & O'Donnell 1997). This technique has been successfully used to measure intracellular [K^+] and [Cl^-], and luminal [Na^+] and [Cl^-], in *Locusta* Malpighian tubule cells (Morgan & Mordue 1983b). However, this method of ion analysis suffers from limited intracellular spatial resolution, perhaps 1-2µm at best, and is restricted to the ionic species selected prior to impalement (Gupta 1989; Sigee *et al.* 1993). It is also difficult to know the position of the microelectrode tip and to place it accurately in narrow extracellular spaces;

mechanical disruption may also occur to such regions as a result of impalement (Gupta 1989). Nevertheless, since ion-selective microelectrodes measure free ion activity, results obtained using this method are valuable in combination with data gathered by other means.

Numerous such complementary techniques are available, including X-ray microanalysis (EXPMA, EPMA or EDS), particle-induced X-ray emission (PIXE), electron energy loss spectroscopy (EELS) and secondary ion mass spectroscopy (SIMS) (Sigee *et al.* 1993). Some of the techniques listed above differ fundamentally in terms of their basic principles and practical aspects. Others are superficially similar, for example PIXE and EPMA (Watt *et al.* 1993; Warley 1997). All differ in their suitability for the analysis of biological tissue, based on their spatial and chemical resolution, ease of quantification, availability, cost and complexity. The use of PIXE and scanning transmission ion microscopy (STIM) has been recently extended to analyse zinc and copper distribution in the entire Malpighian tubule complex of *Drosophila melanogaster* (Schofield *et al.* 1997), but many of the methods described above have significant disadvantages, mainly relating to spatial resolution, quantification and complexity. In this study, X-ray microanalysis has been employed to quantify the elemental content of *Locusta migratoria* L. Malpighian tubule cells, correlating detailed chemical analysis with high resolution morphological information. A vast literature now covers this technique as applied to biological tissue, and it is beyond the scope of this introduction to review this. Nonetheless, appendix one summarises some of the most important aspects of biological X-ray microanalysis, and concludes with a description of the theoretical basis of elemental quantitation as performed in this study. The historical development of quantitative X-ray microanalysis, and its subsequent application to biological tissue has been described in detail elsewhere, for reviews, see Hall (1986, 1989). This introduction is therefore confined to a discussion of the use of X-ray microanalysis in investigations on insect secretory epithelial physiology.

Investigations into insect epithelial physiology using X-ray microanalysis.

A number of previous studies on insect epithelial cells have used X-ray microanalysis to assess the elemental composition of these cells (Gupta 1993), in particular, the Malpighian tubules of *Rhodnius prolixus* (Gupta *et al.* 1976), and

Drosophila hydei (Wessing *et al.* 1986; Zierold & Wessing 1990; Wessing & Zierold 1993, 1996, 1997; Wessing *et al.* 1992, 1993). Pivovarova and co-workers (Pivovarova 1993, 1994a,b) used X-ray microanalysis in conjunction with measurements of the rate of secretion, and cation content of the secreted fluid, to investigate the intracellular elemental content of *Locusta migratoria* Malpighian tubules. A notable feature of these investigations was the use of fluid production stimulators and inhibitors to perturb the normal secretory state of the tubules.

Initial reports on the intracellular elemental content in the upper portion of Malpighian tubules of *Rhodnius prolixus* marked the first application of X-ray microanalysis to the study of insect epithelial cells (Gupta *et al.* 1976). Though earlier biological studies measured the pathological accumulation of copper, iron and tin in tissue (Tousimis & Adler 1963; Lever & Duncumb 1961; Robertson *et al.* 1961), and calcium development in bone formation (Brooks *et al.* 1962), formidable technical difficulties had to be overcome in order to measure the distribution of elements thought to exist intracellularly as diffusible ions, at physiological concentrations (Moreton *et al.* 1974; Hall 1986).

By analysing cryofixed *Rhodnius prolixus* Malpighian tubule cells in both the frozen-hydrated and frozen-dried state, the intracellular concentrations of Na, K and Cl were reported, and demonstrated to be non-uniform across these epithelial cells (Gupta *et al.* 1976). The basal lamina of the tubules displayed a much higher concentration of K than was present in the dissecting saline, and a lower concentration of Na and Cl. A gradient of K was present in the cytoplasmic region, and along the apical microvilli, which increased from basal to apical surface. The concentration rose from approximately 70mM in the basal infoldings to 160mM at the tip of the apical microvilli. The Cl concentration was lowest in the central cytoplasm, but rose at the basal infoldings and the apical cytoplasm; there was a fall in its concentration along the apical microvilli. The concentration of Cl varied from approximately 85mM at the basal infoldings, to 31mM in the central cytoplasm, whilst at the apical tip, the Cl concentration was 68mM. The Na distribution displayed a very similar pattern to that of Cl, though at each site, the concentration of Na was between a half and a third of that of Cl (Gupta *et al.* 1976).

As a result of the use of a cryoprotectant in these experiments, extracellular preservation was sufficient to permit the analysis of regions between the apical microvilli, and within the tubule lumen itself (Gupta *et al.*

1976). Unstimulated tubule cells also showed gradients of Na, K and Cl in the microvillar extracellular spaces, with the concentrations of all three increasing the closer recording were taken to the lumen. Na concentrations in this region were higher than those of the adjacent microvillar cytoplasm, but both K and Cl were lower (Gupta *et al.* 1976). The measurements made in the lumen of Na and Cl concentrations with X-ray microanalysis were confirmed by isotopic measurements of $^{22}Na^{+}$ and $^{36}Cl^{-}$ in the secreted fluid (Gupta *et al.* 1976).

By stimulating fluid production with 5-HT, changes were observed in the cytoplasmic concentration of Na, which increased by approximately 30mM. In the extracellular region beside the apical microvilli, increases in the Na and K concentrations were apparent, of between 10 and 30mM. Increases in the Na and Cl concentration within the apical microvilli were also apparent, with Cl in particular showing substantial increases of between 40 and 90mM (Gupta *et al.* 1976). From the directions of the gradients present in the extracellular spaces, it was concluded that the standing gradient model of solute-water coupling could not be supported in *Rhodnius* tubule cells. Further, it was speculated that the anion deficit in the basal lamina region may have been the result of preferential binding of cations to anionic extracellular organic material (Gupta *et al.* 1976).

X-ray microanalysis was subsequently used to assess the elemental concentration during stimulated and unstimulated secretion in *Calliphora* salivary gland (Gupta *et al.* 1978). It was found that in response to 5-HT, the intracellular elemental concentrations remained relatively constant in spite of large rises in the net movement of ions across the epithelial cells (Gupta *et al.* 1978). A slight drop in the concentration of Na and Cl was seen, and K rose. Na changed from 20 to 15 mM Kg^{-1} w.w., Cl from 33 to 23 mM Kg^{-1} w.w. and K from 115 to 125 mM Kg^{-1} w.w. Stimulation also increased the levels of K in the canaliculi. When the salivary glands were bathed in K^{+}-free saline, the concentration of K fell to a level below that which could be quantitatively analysed, but Na rose to replace K in all the cell regions, and even in the basement membrane (Gupta *et al.* 1978). The basement level of K was raised during stimulation by 5-HT (Gupta *et al.* 1978). High levels of Ca were recorded from dense bodies in *Calliphora* salivary glands, but these were regarded as mitochondrial in nature (Gupta *et al.* 1978).

Taking into account earlier electrophysiological studies in *Calliphora* salivary gland (Berridge *et al.* 1975; Berridge & Schlue 1977) it was concluded that stimulation with 5-HT caused a fall in the intracellular [Cl], as the apical efflux

of Cl^- increased in conjunction with a rise in K^+ efflux (Gupta *et al.* 1978). The resulting hyperpolarisation of V_b was thought to enable the passive influx of K^+ down its concentration gradient. The replacement of K^+ with Na^+ in the tubule fluid under K^+-free incubation conditions was considered to be a simple consequence of a similar change in the cell cytoplasm (Gupta *et al.* 1978). With regard to solute-water coupling at the canaliculi, the evidence suggested that this was a result of local osmosis, with water flow possibly taking place partially transcellularly (Gupta *et al.* 1978). The overall fall in the water content of the salivary gland cells on stimulation with 5-HT (about 5%) suggested that under these conditions, osmotic entry of water would be favoured. These and other findings by Gupta and co-workers were reviewed in Gupta & Hall (1979) and Gupta (1979).

Further studies on Malpighian tubules were carried out in *Drosophila hydei* larval Malpighian tubules using X-ray microanalysis (Wessing & Zierold 1993). A non-uniform distribution of Na, K and Cl was observed. An increasing gradient of K occurred from the basal to apical cell surfaces under control conditions, which was particularly distinct in mmol Kg^{-1} w.w. data (Wessing & Zierold 1993). A similar pattern of distribution of Cl was seen, though the concentrations were lower than K at each analysed area. The basal lamina was characterised by very high levels of all three of these elements. It was commented that the gradient of distribution in K was difficult to explain in light of the mobility of K^+ within the cell (Wessing & Zierold 1993). From the concentrations in K and Cl in the basal labyrinth, the basolateral presence of K^+ channels was inferred (Wessing *et al.* 1986; Wessing & Zierold 1993). These findings were considered to be in general agreement with those in *Rhodnius* Malpighian tubule cells (Gupta *et al.* 1976).

Intracellular and intraluminal mass dense concretions were also observed and analysed using X-ray microanalysis in *Drosophila hydei* (Wessing & Zierold 1992). Two types were present; Type I, rich in Ca, Mg and P, and Type II, chiefly rich in K with lower levels of Mg, Ca, P, S, Cl and Na. (Wessing & Zierold 1992). These differed in their sites of formation but were both seen to be excreted unaltered during development (Wessing & Zierold 1992). By adding Group 2 metals such as Sr and Ba to the animals' diet, Type I concretions were seen to concentrate these elements, and depending on the relative concentrations of Ca, Mg and P in the diet, raised and lowered the concentration of these elements

differentially (Wessing & Zierold 1992). The high concentration of K at the apical surface was later proposed to be the result of an interaction between K and the intravesicular filamentous material which comprised the mass dense concretions (Wessing & Zierold 1996).

An investigation was carried out to examine ion transport in *Drosophila* Malpighian tubules with regard to the effect of bafilomycin A_1, amiloride and Ba^{2+} on intracellular elemental distribution and luminal pH (Wessing *et al.* 1993). This investigation aimed to study ion transport with reference to the model proposed by Bertram *et al.* (1991). Ba^{2+} caused an increase in [K] and [Cl] which was attributed to the blocking of basolateral K^+ conductance (Wessing *et al.* 1993). A continuous "back-flux" of K^+ was thought to occur across the basal membrane. The counterintuitive decrease in K in dry weight terms during inhibition with bafilomycin A_1 was considered to be a consequence of the cell shrinkage and water loss which took place under these conditions (Wessing *et al.* 1993). Bafilomycin was therefore seen to raise the K concentration in wet weight in the basal and central areas of the cell (Wessing *et al.* 1993). Both amiloride and bafilomycin abolished the intracellular K gradient (Wessing *et al.* 1993). Intracellular and luminal pH changes were measured using ISM, and amiloride was seen to lower the luminal pH, as did the use of K^+-free bathing saline (Wessing *et al.* 1993). These data strengthened support for the apical presence of a functionally coupled proton-pumping V-type ATPase and K^+/nH^+ antiporter (Wessing *et al.* 1993).

Brefeldin A, which disrupts the Golgi complex intracellularly, was observed to lower the apical microvillar [K] in terms of the dry weight, and decrease the water content in larval *Drosophila* Malpighian tubule cells (Wessing & Zierold 1996). In no other region of the cell was the K or Cl concentration affected by this agent. The considerable K accumulation in the apical part of the cell under control conditions was therefore found to be due to the glycosaminoglycans (GAG) synthesised in the Golgi-ER complex. As a result, a central role for the GAG in the delivery of K^+ to the apical surface was suggested, and a model incorporating K-binding GAG within Golgi-derived vesicles advanced (Wessing & Zierold 1996). Most recently, the carbonic anhydrase (CA) inhibitors acetazolamide and hydrochlorothiazide were observed to lower basal cytoplasm K, Mg and Cl concentrations measured by X-ray microanalysis in this species (Wessing *et al.* 1997). A basolateral Mg^{2+}/H^+ antiporter in parallel with Cl^-/HCO_3^-

was posited on the basis of these findings (Wessing *et al.* 1997). Cytochemical localisation of CA within the Golgi-ER complex and intracellular vacuoles suggested that this enzyme might be an important source of protons and HCO_3^- for basolateral antiporters and the apical V-type ATPase in larval *Drosophila hydei* Malpighian tubules (Wessing *et al.* 1997).

Investigation of the elemental distribution in *Locusta migratoria* L. Malpighian tubules indicated that K also assumed a gradient intracellularly in this species (Pivovarova *et al.* 1993, 1994a,b). No other element adopted this distribution. Intracellular Cl and Na concentrations were low, the latter below its detection limit throughout the majority of the cell (Pivovarova *et al.* 1994a). The nucleus had a greater concentration of K and Mg than the surrounding cytoplasm, but was slightly less dense (Pivovarova *et al.* 1994a). The basement membrane elemental composition differed considerably from that of the cell, with high Na and Cl, but lower P and K levels (Pivovarova *et al.* 1994a). Intracellularly, the dominant element was P, whose concentration was uniform. The K mimic Rb was detected intracellularly when the tubules of this species were incubated in Rb-containing saline (i.e. K^+-free) *in vivo*, and the K concentration fell dramatically (Pivovarova *et al.* 1993, 1994a). The intracellular levels of Rb assumed a gradient similar to that seen for K, though the sum of the K and Rb concentrations at each site were considerably less than the concentration of K in control incubated cells (Pivovarova *et al.* 1994a). The K concentration in the secreted tubule fluid was nonetheless relatively unaffected by Rb saline, though the rate of secretion was significantly slowed (Pivovarova *et al.* 1994a). Mass dense concretions were also recorded in these cells, and were separated into three groups on the basis of their differing elemental content. Briefly, one type was rich in P and Ca (type A), another in S and K (type B), and a third in Mg and K (type C), but all were observed to replace K with Rb in cells incubated in Rb-containing saline (Pivovarova *et al.* 1993, 1994a). On the basis of this lability, it was proposed that these structures may have acted as intracellular K stores in order to maintain the observed K^+ concentration in the secreted fluid when, for example, the cells were incubated in Rb-containing saline (Pivovarova *et al.* 1994a). It was suggested that the Na^+ and K^+ exited into the lumen via separate mechanisms, and that basally, either all the entry mechanisms for K were able to transport Rb less efficiently than K, or that at least one such mechanism was unable to transport Rb at all (Pivovarova *et al.* 1994a).

Incubation of *Locusta* Malpighian tubule cells in the Na^+-K^+-ATPase inhibitor ouabain initiated a rise in intracellular [Na] and a fall in [K] of approximately 40% at each site, though the gradient of K was retained. No change in the concentrations of P or Cl occurred (Pivovarova *et al.* 1994b). NEM, a V-type ATPase inhibitor, caused an increase in [K] concentration at nearly all cells sites but had no effect on either Na or Cl. The combination of ouabain and Rb-saline reduced the amount of Rb accumulated in the tubule cells, whilst when NEM was used in Rb-containing saline, K was retained though the concentrations were still very much lower than control incubated tissue (Pivovarova *et al.* 1994b). One difference between inhibition with ouabain and NEM in Rb-saline was that Na was present at a higher concentration in the former case (Pivovarova *et al.* 1994b). Clearly, these results supported the apical presence of a proton pumping V-type ATPase and that significant amounts of Rb^+ entered the cells via the Na^+-K^+-ATPase, though the activity of this pump may have been reduced in Rb-saline (Pivovarova *et al.* 1994b).

Aims.

The aim of the preceding short review on the uses of X-ray microanalysis in the study of insect epithelial tissue was to demonstrate that this approach has proved a profitable analytical method, particularly when combined with agents which alter the normal functioning of the epithelia, and supported by data obtained by complementary techniques. This chapter extends the investigations by Pivovarova and colleagues in *Locusta* Malpighian tubules using this approach (Pivovarova *et al.* 1993, 1994a,b), and on the findings described in chapter 3 of this study. By measuring the intracellular concentration of selected elements at various subcellular locations in tubule cells, in the presence of a number of the stimulators and inhibitors of fluid production used in chapter 3, it is hoped to gain additional information regarding ionic transport in these cells. The inhibitors used were 1 mM furosemide, 1 µM bafilomycin A_1 and Rb-containing saline. The stimulators used were 1 mM DB-cAMP and corpora cardiaca extract at a concentration of 0.1 gland pair per ml saline. It was found that Rb did not fully replace K intracellularly in *Locusta* Malpighian tubules (Pivovarova *et al.* 1994a), yet that this element appeared in the mass dense concretions present in the cell cytoplasm. By combining microanalysis data with measurements of the cation

composition and rate of fluid secretion in Rb-containing saline, it was suggested that a common efflux site for Na^+ and K^+ was unlikely, and that Rb entry basolaterally may have been via the action of the Na^+-K^+-ATPase (Pivovarova *et al.* 1994a). A possible role for the dark bodies as a site of K storage was also tentatively advanced (Pivovarova *et al.* 1994a). Confirmation for these finding is sought here.

A similar rationale has been employed to assess the effect of furosemide on the tubule cells. The effect of furosemide on intracellular elemental distribution is unknown in insect Malpighian tubule cells, though bumetanide and Rb-containing saline were used in conjunction by Dörge *et al.* (1989) to confirm the presence of a basolateral Na^+-K^+-$2Cl^-$ cotransporter in abdominal skin from *Rana temporaria*, using X-ray microanalysis (Dörge *et al.* 1989). The cotransporter was able to substitute Rb for K but was inactive under control conditions. Additionally, Na^+-K^+-$2Cl^-$ cotransport was investigated using amiloride and Rb in amphibian epithelial tissue using X-ray microanalysis (Dörge & Rick 1990).

Bafilomycin A_1 has been used by Wessing and co-workers in a study on larval *Drosophila hydei* Malpighian tubules (Wessing *et al.* 1993), as well as the carbonic anhydrase inhibitors acetazolamide and hydrochlorothiazide (Wessing *et al.* 1997). Their results supported the theorised V-type ATPase / K^+ antiporter suggested by Bertram *et al.* (Bertram *et al.* 1991). Bafilomycin caused a drop in luminal pH as measured by ISM, plus a substantial decrease in the water content of the cells, especially in the basolateral region. However, these authors also reported a decrease in the basal K content in terms of dry weight (Wessing *et al.* 1993). It is intended to follow up this unexpected finding in *Locusta*.

DB-cAMP was demonstrated to increase the rate of fluid production in *Locusta* Malpighian tubules in the previous chapter. The effect on intracellular elemental distribution of exogenous cAMP is also unknown in Malpighian tubule cells, but has been examined with quantitative X-ray microanalysis in human sweat gland cell (Mörk & Roomans 1993). The Cl and K content in these cultured cells both decreased when the cells were stimulated with 5 mM 8-bromo-cAMP. It has been suggested that cAMP raises intracellular Cl in tubule cells (K. Bowler, pers. comm.), and further information is sought regarding the presence of the possibly cAMP-stimulated basolateral Na^+-K^+-$2Cl^-$ cotransporter.

Stimulation of *Rhodnius* Malpighian tubules with 5-HT raises intracellular Na and Cl (Gupta *et al.* 1976; Gupta & Hall 1979), but this may not be reflected in a similar result in corpora cardiaca stimulated *Locusta* tubules, due to the predominantly K^+-rich nature of its luminal fluid of the latter species when stimulated (Al-fifi *et al.* 1998b). *Rhodnius* secrets a fluid with similar concentrations of Na^+ and K^+ post-blood meal (Maddrell 1969). As well as measuring the intracellular elemental concentration as a consequence of the action of both cAMP and corpora cardiaca extract , the content of the enigmatic cytoplasmic mass dense concretions has been examined. This is of special interest, in light of their apparent lability and the their hypothesised role in the cellular ionic economy of *Locusta* tubules (Pivovarova *et al.* 1994b).

4.2. Methods.

In order to obtain qualitative and quantitative X-ray microanalysis data from frozen tissue, a comprehensive calibration process was completed. The purpose of section 4.2 is to summarise briefly this calibration procedure. Sections 4.2.2 and 4.2.3 were performed according to the manufacturer's manual (Quantem/FLS Operators Manual SR2-500-QMF-0283).

4.2.1. System settings.

The following settings were entered into the Quantem-FLS program initially and unless otherwise stated, remained constant for the entire period of the study. The first five values refer to mechanical settings on the electron microscope in itself and the remainder to Quantem-FLS program parameters essential to analysis.

(i) Tilt (of specimen) = 12°.
(ii) Elevation = 0 (default value).
(iii) Azimuthal angle = 0 (default value).
(iv) Cosine = 1.000
(v) kV = 100
(vi) Low keV limit = 4 keV (for continuum measurement).
(vii) High keV limit = 16 keV (for continuum measurement).

(viii)Grid element = nickel.

(ix) eV/channel = 20 eV.

(x) Peak energy = 7.4710 keV (peak centre for Ni).

(i) refers to the angle of tilt from the horizontal of the specimen about the eucentric axis of the electron microscope specimen stage. The holder and grid were thus tilted towards the detector as shown in Figure 4.1. (vi) and (vii) defined the area from which the continuum or background counts were measured to enable elemental quantification by the continuum normalisation or the Hall method (Hall 1971). The holder element option was defaulted since a beryllium holder was used (Warley 1997).

4.2.2. Zero-strobe peak setting/ noise elimination.

The aim of this procedure was to assess the effect on the detector and pulse processor of changes in the count rate. The system noise was minimised by setting the fast and slow potentiometers to give count rates of 50 counts per second greater than their lowest values; the zero-strobe (or noise) peak was then set to its lowest possible value and re-centred on zero as necessary.

4.2.3. System resolution setting.

A Formvar-coated nickel grid was used to collect a series of spectra in which the dead-times ranged from 10 to 50%. These spectra were processed within an appropriate part of the Quantem-FLS program, and the resolution of the system calculated as the largest FWHM (full width half-maximum) value of the Ni K peaks of all the derived spectra, **plus** 4eV. The system resolution was therefore set at 123eV, and remained set at this value for rest of the study.

4.2.4. Profile preparation.

Profile spectra were collected to define the correct shape and position of the characteristic peaks of the elements to be analysed. Both bulk standards (Roomans 1979; Warley 1997) and sprayed microdroplets were used (Pivovarova *et al.* 1994a,b; Warley 1997). For Fe, Al, Ni and Ca, bulk standards

were analysed at 20 keV. The use of this voltage was essential to minimise the absorption steps produced by bulk-type standards. The spectra thus generated were processed within part of the Quantem-FLS program that compensated for this lower probe voltage; note that for the rest of this study, microanalysis was carried out using a probe voltage of 100 kV. Two to four bulk standard spectra were averaged before processing. Na, Ca, K, Rb, Mg, Cl, Mn, and P profiles were prepared by spraying microdroplets of a suitable salt solution onto Formvar-coated nickel grids, using a small hand-held atomiser (Warley 1997). The salts used were NaCl, RbCl, $CaCl_2$, KH_2PO_4, $MnSO_4$, $MgSO_4$ and NaH_2PO_4. At least 30 to 40 microdroplet spectra were taken for each element, averaged, and processed in Quantem-FLS. Care was taken to ensure the peaks obtained by both methods were "clean", that is, within the correct energy channel and that they displayed the correct shape.

4.2.5. Quantitative (intensity) calibration.

The ultimate aim of this part of the calibration procedure was to analyse a frozen-dried section in exactly the same manner as a cryofixed Malpighian tubule cell, but which was composed of a protein matrix plus a known amount of one of 3 cations, namely Na^+, K^+ and Rb^+. Cryofixed solutions of 20% BSA (bovine serum albumin) plus an appropriate salt were prepared (Dörge *et al.* 1974). The salt solutions used were KH_2PO4, NaCl (both 100mM) and RbCl (200mM). The concentrations of the solutions were verified using a Perkin-Elmer 5000 atomic absorption spectrophotometer. Na^+ and K^+ concentrations were measured using absorption at 589 and 766.5nm respectively; Rb^+ was analysed on emission at 780nm. Table 4.1 shows these data.

Table 4.1. The calculated and measured ionic concentrations of standard solutions used in intensity calibration (mean ± SEM).

Cation	Calculated concentration (mM)	Measured concentration (mM)	n
K^+	100	101.37 ± 2.53	11
Na^+	100	93.25 ± 4.20	11
Rb^+	200	202.40 ± 8.05	6

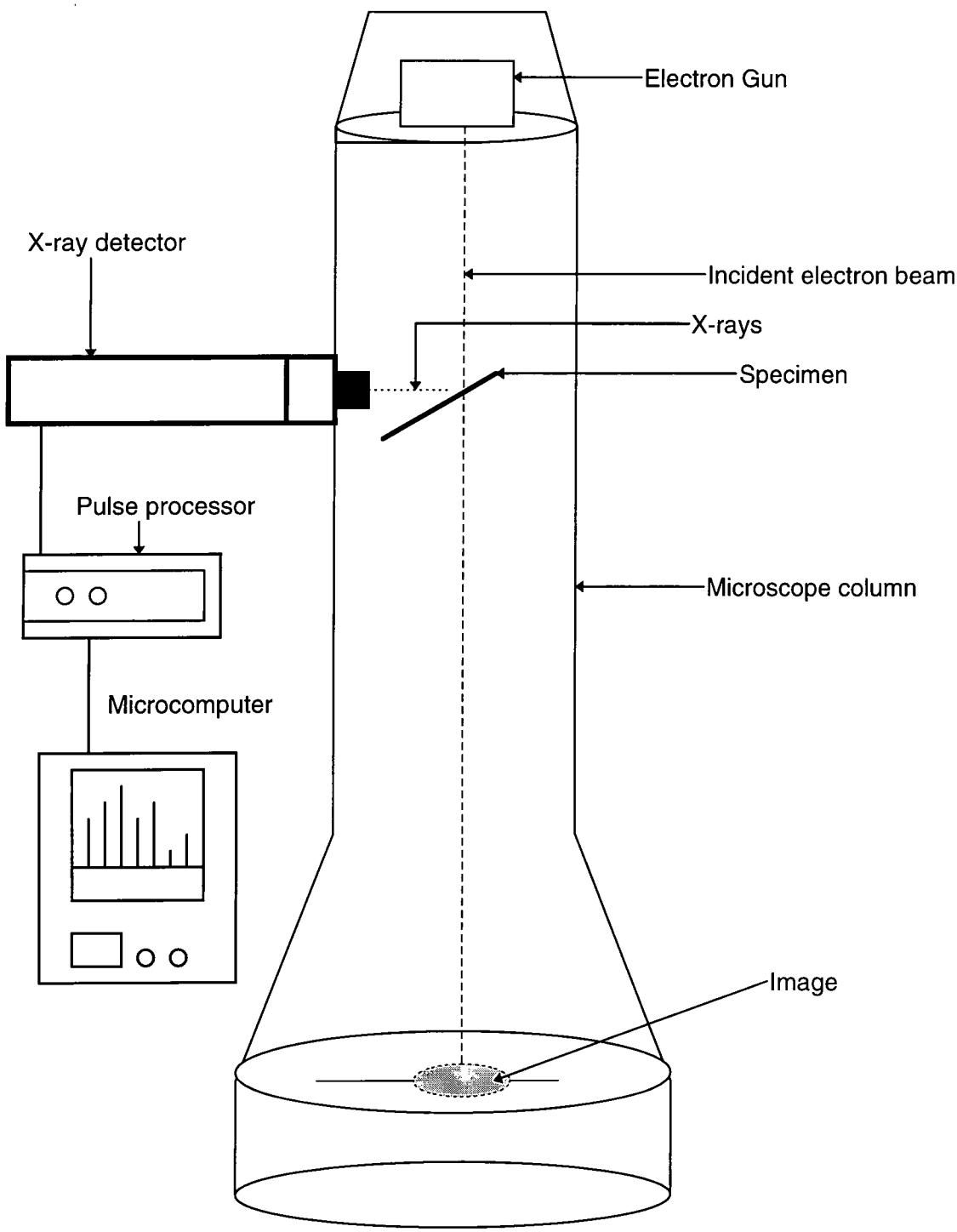


Figure 4.1. Schematic representation of the combined electron microscope/microanalyser used in this study. Briefly, incident electrons pass down the microscope column and through the frozen-dried specimen. The X-rays generated are detected by the X-ray detector, and signals processed by the pulse processor before being recorded as the spectra on the microcomputer screen. The specimen is tilted 12 degrees towards the detector window to increase the efficiency of X-ray photon detection.

The solutions were used to make up a 20 % solution of dialysed bovine serum albumin (BSA). The BSA was dialysed over 24 hours in distilled, polished water before freeze drying (Roomans & Seveus 1977). The aim of dialysis was to remove all possible ionic contamination from the BSA. The standard solution plus 20% BSA thus acted as an artificial "cell" with a precisely known composition.

Cryofixation of the 20% BSA solution was performed as described in section 4.2.7. Since the frozen solutions were more brittle than cryofixed biological material, sections were cut with the knife and specimen maintained above -100°C (i.e. warmer).

Once carbon-coated, these sections were analysed to calculate correction values for K, Na and Rb, known as the FST values. These correction factors were used by the quantitation program to calculate the elemental concentrations in cryofixed biological material of unknown composition. 10 to 20 spectra were generated for each standard solution, and an FST value for each derived from the Quantem-FLS program. These values were averaged to give the final FST values for K, Na and Rb. A comparison with the FST values generated from the calibration of this system, and those reported by Warley in an earlier study may be found in Table 4.2 below (Warley 1990). It is clear that the Na and K FST values displayed are in close agreement.

Table 4.2. The FST values for K, Na and Rb calculated in this study (mean ± SEM). (Published data column refers to Warley, 1990).

Element	FST value	Published data
Na	0.000736996 ±0.00017005 (n=27)	0.0007226 ±0.000120
K	0.004949986 ±0.000675043 (n=7)	0.0047810 ±.000794
Rb	0.00459695 ±0.001112931 (n=21)	(Not available)

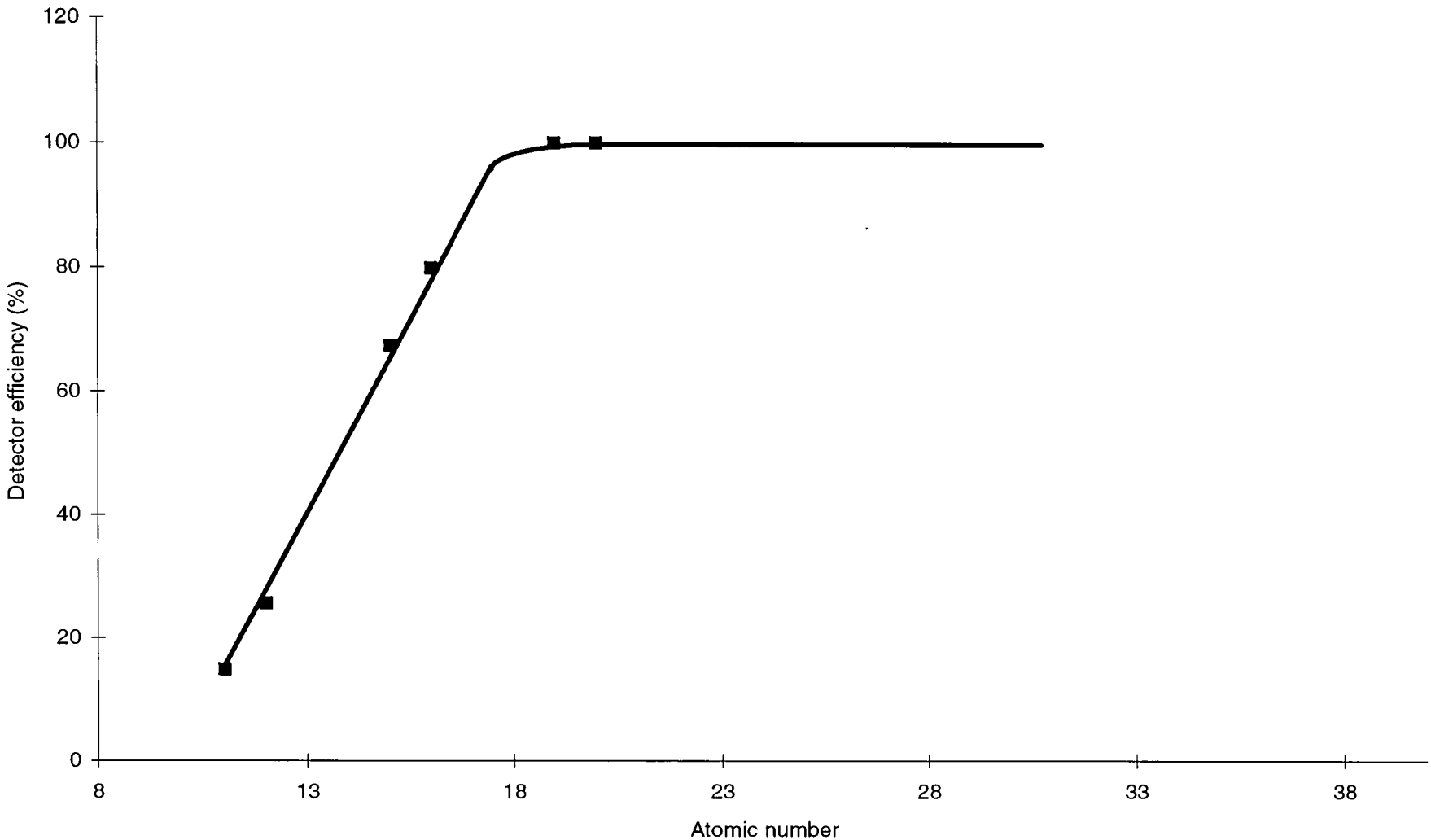
To determine the FST values of Mg, P, S, Ca, Cl and Rb, the relative detector efficiency method was applied (Morgan *et al.* 1975). Various factors affect the number of counts detected for each element, so that even in an isoatomic solution, two different elements will not produce the same number of characteristic counts. Hence, they will have different areas under their characteristic peaks. It is therefore possible to calculate the relative detector efficiency for each element by normalising the data to one element, which in this calibration procedure, was potassium (Warley 1997).

An isoatomic solution of K, Na, P, S, Ca, Cl and Mg was prepared using the following salts; $MgSO_4 \cdot 7H_2O$, NaCl, KH_2PO_4 and $Ca(NO_3)_2 \cdot 4H_2O$ (Morgan *et al.* 1975; Warley 1997). The solution was sprayed onto a Formvar-coated nickel grid and allowed to air dry. Spectra were collected from the droplets as soon as they had dried, in the same manner as for the collection of profile spectra (section 4.2.4). A total of 20 spectra were produced from separate droplets and the area under each element's characteristic peak averaged and normalised to K (i.e. K=100% efficiency). A graph of relative detector efficiency was then drawn (see Figure 4.2). From this graph, FST values for chlorine and rubidium were calculated. Table 4.3 details all the FST values used in this study.

Table 4.3. The FST values for all elements analysed in this study and their derivation. Calculated values were derived from the relative detector efficiency graph.

Element	FST	Relative efficiency (%).	Source
Na	0.0007370	14.89	frozen std.
K	0.0049450	100.00	frozen std
Mg	0.0012796	25.68	isoatomic droplet
P	0.0033366	67.41	isoatomic droplet
S	0.0039539	79.88	isoatomic droplet
Ca	0.0049733	100.47	isoatomic droplet
Rb	0.0049995	101.00	calculated
Cl	0.0044055	89.00	calculated

Figure 4.2. Relative detector efficiency graph.



4.2.6. Preparation of cryofixed Malpighian tubule cells for X-ray microanalysis.

Once the calibration procedure described in sections 4.2.1 to 4.2.5 was completed, cryofixed tubule cells were prepared. Section 4.2.6 explains the steps required to prepare experimental tissue for analysis.

Approximately equal numbers of adult sexually mature male and female locusts were sacrificed as described in chapter 2 (Pivovarova *et al.* 1994a,b). For experiments using corpora cardiaca extract, only female insects were used. Experiments using cAMP were carried out on both males and females. The tubules were quickly dissected free from the midgut in control physiological saline (at 30±1°C) and briefly stored in the same saline until sufficient tubules had been obtained, typically from two to four animals. The tubules were then transferred to experimental saline and incubated for 30 minutes, also at 30±1°C. The saline solution was occasionally gently shaken during this period, to permit adequate oxygenation of the tissue.

Immediately after this period, the tubules were plunge-frozen in liquid Arcton-22 (chlorodifluoromethane), on aluminium or brass support plugs (Zierold 1993; Pivovarova *et al.* 1994a,b). The frozen tubules were cryosectioned with dry glass knives (clearance angle 4°) on an RMC MT 6000 ultramicrotome with FS 1000 cryosectioning attachments. Sections were cut at a thickness of between 100 and 300nm. Knife and specimen temperatures varied between -90 and -130° C. Sectioning speeds were between 1.0 and 1.4 mm s^{-1} (Pivovarova *et al.* 1994a,b). Ribbons of sections were only infrequently produced. Usuable sections, i.e. those almost transparent in appearance, were usually only obtained from approximately the uppermost 8µm of the specimen. Beyond this point, the sections took on a much whiter appearance and were easily fragmented, probably as a result of the formation of large ice crystals within the tissue (Warley 1997).

Sections were lifted from the glass knife with a clean, precooled eyelash probe and transferred onto a precooled Formvar-coated nickel parallel bar grid (Warley & Gupta 1991; Pivovarova *et al.* 1994a,b). When sufficient sections had been produced, a second precooled grid was placed on top of the first. The two grids were pressed gently together using a precooled brass rod, to flatten and firmly attached the sections to the Formvar coating (Warley & Gupta 1991). After

being separated, both grids were stored in a specially-made brass container within the cryoultramicrotome chamber, whilst further grids were prepared. This container remained partially submerged in liquid nitrogen.

When it was no longer possible to obtain adequate sections from the cryofixed block of tubules, the brass container (whilst still partially submerged in liquid nitrogen) was quickly transferred to an Edward model EPTD vacuum freeze drier. The tissue was dried overnight at approximately $10^{-1.5}$ torr, at ambient temperature. Subsequently, the grids were carbon-coated in either an Edwards model E12E or model 12E6/1288 carbon coater (Pivovarova *et al.* 1994a).

4.2.7. Elemental analysis.

The elemental composition of the tubule cells on the carbon-coated grids was quantified using a Philips EM 400T transmission electron microscope equipped with a retrofitted Link Analytical AN10000 energy dispersive X-ray microanalysis system. The Quantem-FLS (filtered least squares) program quantified the presence of Na, K, P, S, Cl, Mg, Rb and Ca according to the continuum normalisation method or Hall method (Hall 1971; Hall 1979; Hall & Gupta 1982). All the elements were analysed by measuring the area under the their Kα peak , apart from Rb, which was quantified from its Lα peak. Continuum radiation was collected over the range 4 to 16 keV. Sections were examined at magnifications of 10-17000 times, with a spot size of 4 and probe diameter was 0.3 to 0.5μm. Live counting time was between 60 and 100s. Probe currents varied from approximately 0.3 to 1.7 nA The probe current was estimated according to the equations (personal communication from Philips Analytical);

Equation 4.1.

$$\mathbf{Is \approx Ip}$$

where;

Is = probe current.

Ip = screen current (both nA).

Equation 4.2.

$$Is = [(53.27s/t-1)(1.687\times10^{-11} + 3\times10^{-11})$$

where;

t = exposure time (s).

s = emulsion setting (=8).

The probe current could thus be estimated from the reading on the plate camera exposure-time meter.

Minimum detectable elemental concentrations were calculated according to the method described by Warley (Warley 1997), using equation 4.3 below;

Equation 4.3.

$$Cm = [3\ Cx\ (Bx)^{\frac{1}{2}}] / Px$$

where;

Cm = minimum detectable concentration of element x.

Cx = known concentration of element x in the specimen spectra.

Bx = number of counts in the background under the characteristic peak of element x.

Px = number of counts in the characteristic peak of element x.

The detection limit for Na was therefore approximately 95 ± 10 mmol Kg^{-1} dry weight, and for other elements in the order of 10 mmol Kg^{-1} dry weight.

Each time a new grid or new section was analysed, a series of correction spectra were obtained. A single spectrum was first recorded near to a grid bar to correct for gain changes in the system. Three further spectra were then taken from different parts of the grid but in close proximity to the grid bars. Averaged, these spectra served to determine the fraction of the continuum derived from the grid. Three more spectra were recorded, from an area near to the section to be analysed, but with the probe beam positioned centrally between the grid bars. These were averaged and used to correct for the continuum derived from the support film.

The sections were then analysed. Mass loss during microanalysis of frozen-dried sections was inevitable (Warley 1997) but care was taken to retard such loss by using the liquid nitrogen cold-trap integral to the electron microscope in order to maintain section temperature at approximately -180°C (Hall & Gupta 1974). Experience showed that prolonged storage of the carbon coated sections resulted in contamination and deterioration of the specimen (also see Warley 1997). Consequently, sections were analysed as soon as practicable after preparation. Repeated analysis of a section, or area of a section, was avoided since it was clear that mass loss sometimes affected elemental quantitation. Some volatilisation of chlorine was observed, though infrequently, and usually in areas which had been already analysed. Data collected from such areas was discarded.

4.2.8 Statistical analysis.

Statistical analysis was carried out to ensure the elemental analysis results obtained from tissue incubated in control saline were normally distributed. Kolmogorov-Smirnov normality tests were used (see appendix two). Comparisons between the means of elemental concentrations were performed using Student's t-tests. Data were tested for equality of variance using F_{MAX} tests. In the following sections, when the results from more than one test are included in brackets, Student's t-test data are presented in the order basal microvilli, basal cytoplasm, central cytoplasm, apical cytoplasm and apical microvilli, unless stated otherwise. The symbol ‡ indicates that the concentration of that element, at that site within the cell, was significantly different from the concentration of the same element at the same site, in tissue incubated in control saline. This does not imply a significant difference between adjacent sites within the same tissue treatment. Where a mean elemental concentration was below the detection limit of that element, no statistical tests have been performed, the mean value being treated as indistinguishable from zero.

The mass density of the electron dense concretions present intracellularly was estimated by calculating the mean background counts from 6 separate energy channels in the spectra generated by the concretions. The channels selected were well away from any characteristic peaks present in the spectra. The means number of counts in each channel, calculated from up to 10

concretions for each dark body type, was subsequently compared with the counts from the same channels in spectra recorded from adjacent cytoplasmic areas. The enabled the derivation of a ratio of mass dense concretion counts to cytoplasm counts in each of the six channels, and the mean of these ratios represented the overall mass density value for that concretion type. It was assumed that the cytoplasm as a whole displayed a uniform mass density.

4.3. Results.

4.3.1. Electron micrographs of conventionally prepared and cryofixed Malpighian tubule cells.

Biological X-ray microanalysis requires the ability to locate ultrastructural features in cryofixed material. Only then can the section be orientated so that the electron beam is able to irradiate a known region. Cryofixed tissue rarely displays the same level of ultrastructural detail as can be resolved in osmium-fixed, resin embedded material. Nevertheless, in good sections of frozen-dried tissue, sufficient detail is usually present to enable microanalysis to be performed (Gupta 1979). The electron micrographs shown in Figure 4.3 illustrates the regions from which elemental concentration readings were taken, in sections fixed and stained for normal electron microscopy, and in the cryosections necessary for quantitative X-ray microanalysis.

4.3.2. Intracellular elemental concentrations in Malpighian tubule cells incubated in control saline.

Table 4.4 reports the elemental concentrations of the seven elements routinely measured in tubules incubated in control saline. The data presented were obtained from several different cells. Statistical analysis (appendix two) demonstrated that the intracellular elemental concentration data were normally distributed, with the exception of Ca concentrations. This is likely to be because Ca occurred in low concentrations, remaining close to its detection limit throughout this study. The basement membrane elemental concentrations also showed a largely non-normal distribution. This may be a result of the highly heterogeneous nature of the acellular membrane and the inevitable retention of

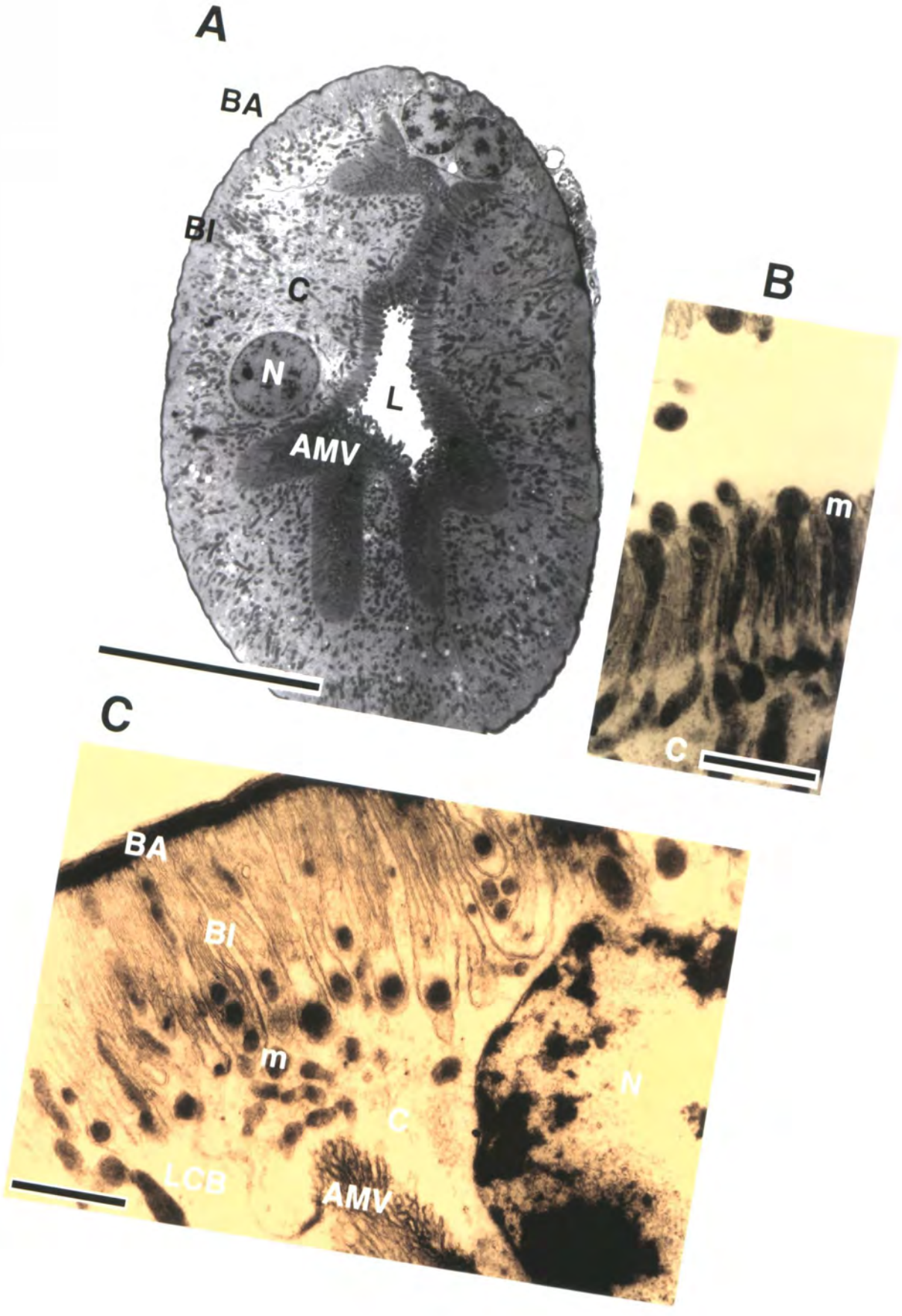
Figure 4.3. Electron micrographs showing ultrastructural aspects of conventionally-fixed and cryofixed *Locusta* Malpighian tubules. For convenience, the following abbreviations label the micrographs.

Abbreviation	Cell site/organelle
BA	Basement membrane
BI	Basal infoldings
C	Cytoplasm
AMV	Apical microvilli
m	Mitochondria
N	Nucleus
L	Lumen
DB	Mass dense concretion
c	Site of loss of mass dense concretion
LCB	Lateral cell border

(A). Electron micrograph of *Locusta migratoria* L. Malpighian tubule in cross section. Conventional fixation. Magnification was 1000 times, bar = 50µm. A dark basement membrane surrounds the outside of the tubule, and situated below, the basal infoldings. The cytoplasm contains numerous mitochondria and in this section, three nuclei are also present. The extensive apical microvilli border is visible adjacent to the tubule lumen. At the upper right of the micrograph, muscle tissue and tracheae are present, on the outer side of the basement membrane. Some intracellular tracheae are visible, for example immediately below the two nuclei at the upper centre of the section; these nuclei may be present in a single (hence binucleate) cell.

(B). Electron micrograph of apical microvillar region. Magnification was 8000 times, bar = 2µm. Conventional fixation. The apical microvilli contain mitochondria, as does the apical cytoplasm immediately below.

(C). Electron micrograph showing basal infoldings and cell cytoplasm. Magnification was 8000 times, bar = 2µm. Conventional fixation. The basement membrane visible at the upper left of the micrograph rests upon the extensive basal infoldings present in this cell. Mitochondria are present in the lower part of


A
BA
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AMV
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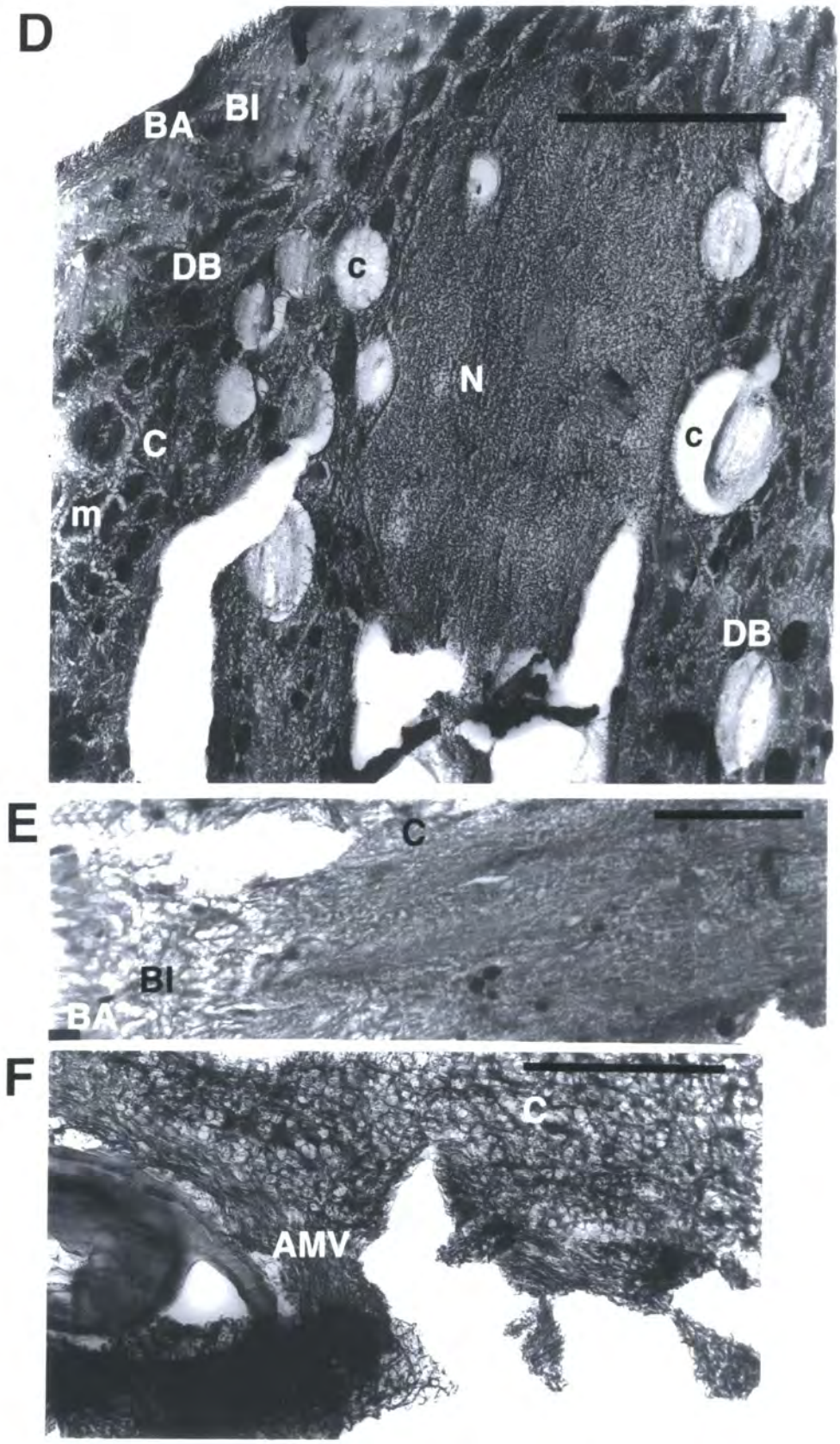
m
C
C
BA
BI
m
C
N
LCB
AMV

the infolding region, and are also observed within the somewhat truncated cytoplasm. A lateral cell border joins with the apical microvilli just visible in the lower centre of the photograph. A nucleus is present at the right of the photograph.

(D). Electron micrograph of *Locusta* Malpighian tubule basal and central cytoplasmic regions. Magnification was 4600 times, bar = 10µm. Cryofixed tissue. Basal membrane visible is visible in upper left of the micrograph, and below, the basal infoldings (slightly indistinct in appearance). The basal cytoplasmic region below this contains mitochondria and a number of mass dense concretions. Also visible are empty, roughly circular holes in the section, believed to contain mass dense concretions which have been lost during sectioning. A large nucleus is present in the centre of the micrograph.

(E). Electron micrograph of *Locusta* Malpighian tubule basement membrane, basal infoldings, basal cytoplasm and central cytoplasm. Magnification was 6000 times, bar = 5µm. Cryofixed tissue.

(F). Electron micrograph of apical cytoplasm, apical microvilli and luminal area. Magnification was 8000 times, bar = 5µm. Cryofixed tissue. At the centre left of the micrograph, a protozoan parasite *Malameoba locusta* is present intraluminally. Apical microvilli are visible beside the parasite.


D
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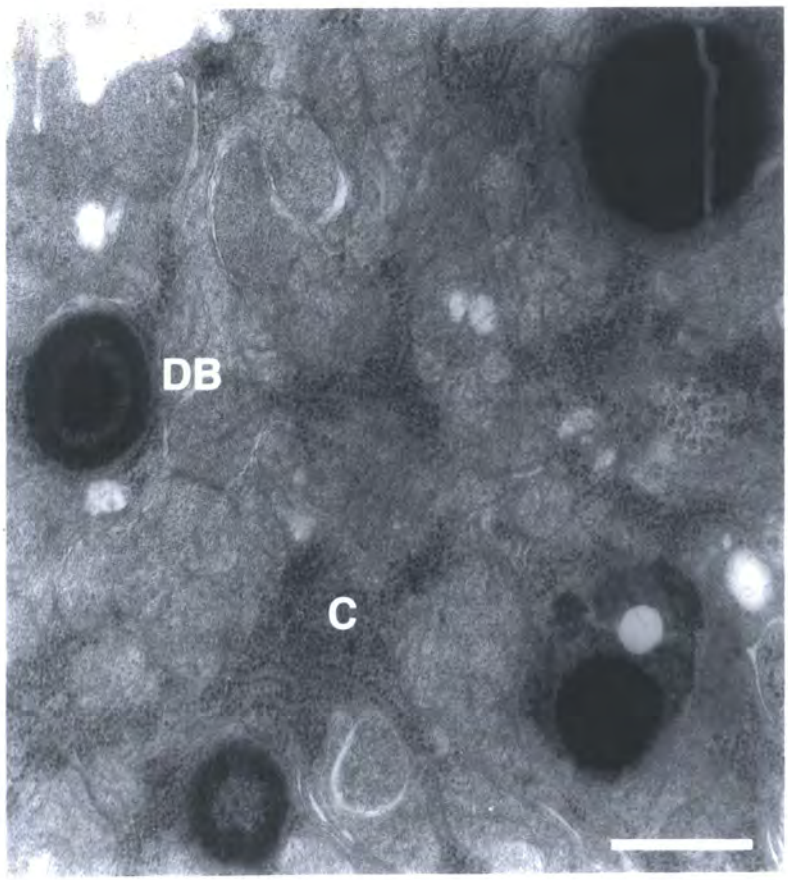
AMV

(G). Electron micrograph of cytoplasmic region containing four mass dense concretions with varying morphology. Magnification was 22000 times, bar = 1μm. Conventional fixation The concretion at the upper right of the micrograph has a presumed sectioning artefact running through it, a vertical split. At the upper left, the concretion displays a laminate appearance, as does the concretion at the lower left.

(H). Electron micrograph of two mass dense concretions. Magnification was 28000 times, bar = 1μm. Again, the variation in the morphology of these structures in clear, and the concretion at the lower left of the micrograph may be situated within a vacuole. Note the comparable size of the concretions and many of the neighbouring mitochondria.

G



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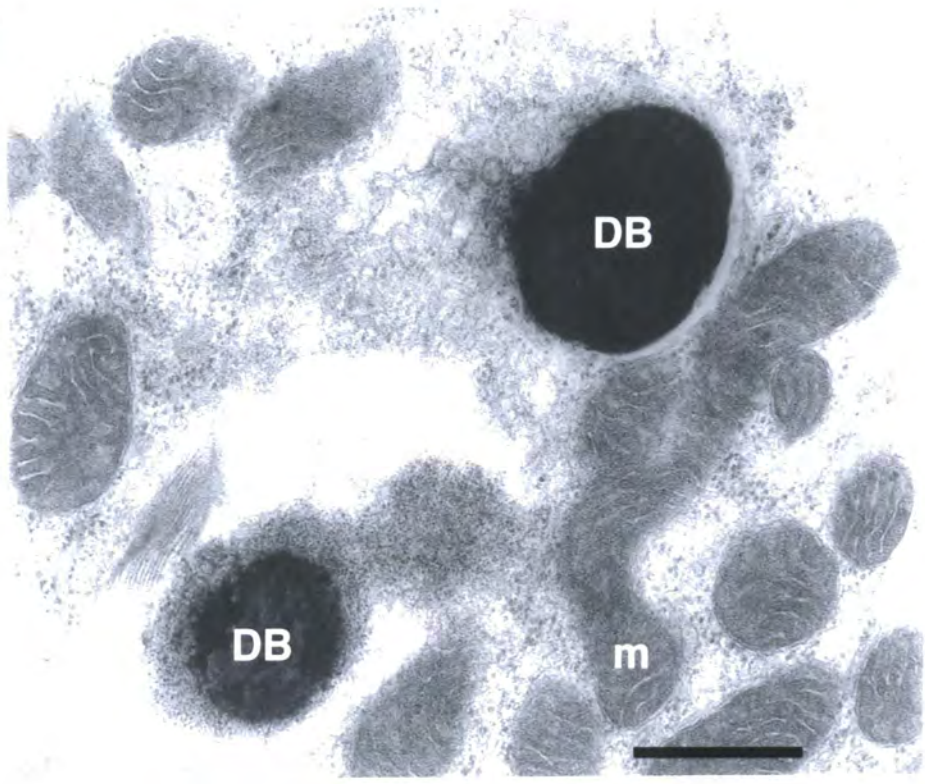


Table 4.4. The elemental concentrations in *Locusta* Malpighian tubule cells after incubation in control saline for 30 minutes. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean.* denotes elemental measurement below the detection limit for that element.

		Elemental concentration (mmol Kg^{-1} dry weight)						
Cell site	*n*	K	Na	Cl	P	S	Mg	Ca
Basement membrane	202	97.6±4.2	533.4±17.4	480.3±16.3	70.5±5.5	170.9± 6.9	77.4± 3.8	32.2± 2.1
Basal infoldings	84	192.8±12.4	294.2±18.4	152.3±11.3	502.4±25.9	92.0± 7.5	81.2± 9.1	*
Basal cytoplasm	74	257.1±25.1	237.1±16.7	109.9±10.2	532.3±32.5	60.6± 9.6	82.8± 7.2	18.9± 5.1
Central cytoplasm	129	347.8±11.1	172.4±16.0	86.5±8.5	613.5±17.8	68.3± 3.3	82.5± 5.6	18.2± 4.3
Apical cytoplasm	24	478.1±32.8	*	128.1±17.5	730.2±43.7	51.0± 4.2	91.9± 17.2	21.6± 8.6
Apical microvillar border	33	480.8±36.1	*	166.6±27.0	638.6±50.3	76.9± 7.1	87.0± 11.5	17.7± 6.2

small areas containing bathing saline at the cell-"haemolymph" interface. As a consequence, Ca concentrations were not compared statistically with other elements. Non-parametric statistical tests were applied to compare basement membrane elemental concentrations where appropriate.

Figure 4.4 illustrates six typical spectra recorded from cryofixed Malpighian tubule cells incubated for 30 minutes in control saline; one spectra is present from each of the six cell sites routinely measured in this tissue. The spectra show that intracellularly, the elemental "signature" of the Malpighian tubule cells altered little, the main changes being in the height of the elemental peaks, especially of K. Conversely, the basement membrane had a noticeably different pattern of peaks, since in this region Na and Cl, and to a lesser extent S, predominated. The mean Na concentration in this region was approximately 533 mmol Kg^{-1} d.w., and the Cl concentration was approximately 480 mmol Kg^{-1} d.w. The S concentration was lower, approximately 171 mmol Kg^{-1} d.w. Table 4.4 shows that Na and Cl are present in the approximate ratio 1:1 (R^2=0.3024, P<0.001). The high concentrations apparent of Na and Cl are likely to be a consequence of the contact between the basement membrane and the NaCl-rich control saline. P, Mg and K are also present in the basement membrane.

The region of the basal infoldings were characterised by an increase in the K concentration of nearly 100 mmol Kg^{-1} compared with that of the basement membrane. The P concentration was also greater than in the basement membrane, though the Na, Cl and S concentrations were lower. The Mg concentration was little changed. The Ca concentration fell below its detection limit. An intracellular concentration gradient of K was clearly present. The concentration increased by about 300 mmol Kg^{-1} d.w from basal to apical surface. At all the sites measured throughout the cell, except the apical cytoplasm and the apical microvilli, the K concentration was significantly higher than at the preceding site. The basal cytoplasm concentration (257.1 ± 25.1 mmol Kg^{-1} d.w.) was significantly higher than that of the basal infoldings (192.8 ± 12.4 mmol Kg^{-1} d.w.) (t=3.572, P<0.001), the central cytoplasm (347.8 ± 11.1 mmol Kg^{-1} d.w.) concentration was higher than the basal cytoplasm (t=5.318, P<0.001), and the apical cytoplasm (478.1 ± 32.8 mmol Kg^{-1} d.w) higher than the central cytoplasm (t=3.761, P<0.001). No significant difference in the potassium concentrations between the apical cytoplasm and the apical microvilli (480.8± 36.1 mmol Kg^{-1} d.w) was found (t=0.054, P>0.05).

Figure 4.4. Six typical spectra recorded from tubule cells incubated in control saline. Each spectra is from one of the six sites routinely analysed in cryofixed tubules. All units are in mmol Kg^{-1} dry weight. BA 399, BMV722 are abbreviations referring to the area from which the spectra was recorded. Vertical axis represents total counts, horizontal axis represents energy channels (keV). The scale of the vertical axis varies. BA = basement membrane. BMV = basal infoldings. CYT = cytoplasm. AMV = apical microvilli.

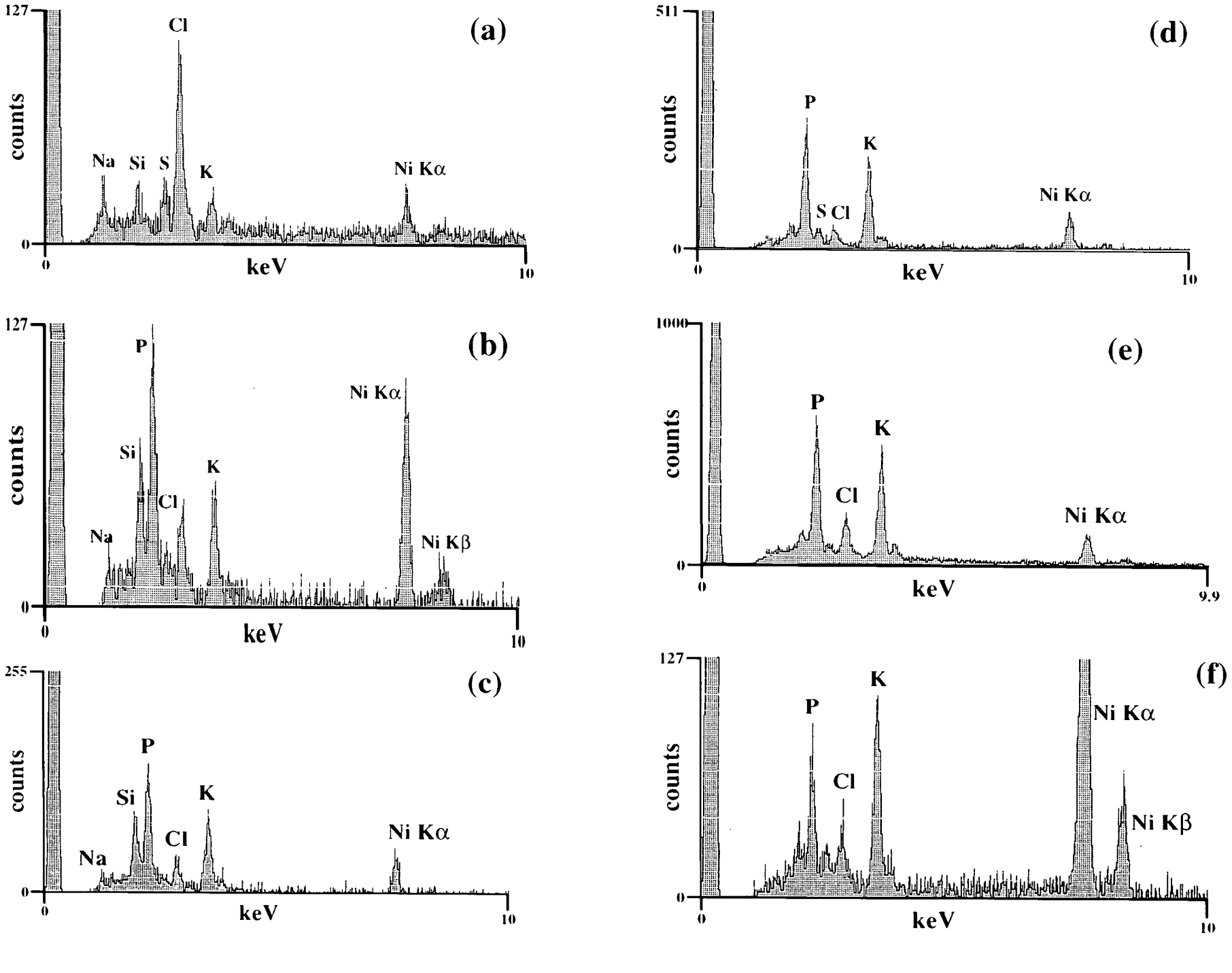
(a). Spectra BA 399. Peaks (from left to right), Na Kα, Si Kα, S Kα, Cl Kα, K Kα and Ni Kα. Elemental concentrations were; [Na] = 553.7, [K] = 78.1, [P] = 0, [S] = 115.7, [Cl] = 471.5, [Mg] = 57.8, [Ca] = 25.7 .

(b). Spectra BMV 722. Peaks (from left to right), Na Kα, Si Kα, P Kα, S Kα, Cl Kα, K Kα and Ni Kα and Ni Kβ. Elemental concentrations were; [Na] = 295.0, [K] = 166.4, [P] = 496.7, [S] = 53.3, [Cl] = 138.0, [Mg] = 0, [Ca] = 9.5 .

(c). Spectra CYT 795 (basal cytoplasm). Peaks (from left to right), Na Kα, Mg Kα, Si Kα, P Kα, Cl Kα, K Kα and Ni Kα. Elemental concentrations were; [Na] = 204.5, [K] = 248.2, [P] = 518.4, [S] = 25.0, [Cl] = 108.1, [Mg] = 89.5, [Ca] = 6.1 .

(d). Spectra CYT 1054 (central cytoplasm). Peaks (from left to right), P Kα, S Kα, Cl Kα, K Kα and Ni Kα. Elemental concentrations were; [Na] = 40.5, [K] = 341.5, [P] = 603.7, [S] = 70.4, [Cl] = 60.1, [Mg] = 80.5, [Ca] = 3.9 .

(e). Spectra CYT 1397 (apical cytoplasm). Peaks (from left to right), Si Kα, P Kα, Cl Kα, K Kα, K Kβ and Ni Kα. Elemental concentrations were; [Na] = 147.2, [K] = 480.9, [P] = 755.0, [S] = 40.5, [Cl] = 186.8, [Mg] = 64.5, [Ca] = 5.0 .


(a)
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Na
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keV
10

(f). Spectra AMV 13. Peaks (from left to right), Si Kα, P Kα, S Kα, Cl Kα, K Kα, K Kβ, Ni Kα and Ni Kβ. Elemental concentrations were; [Na] = 49.7, [K] = 536.4, [P] = 424.1, [S] = 89.3, [Cl] = 149.2, [Mg] = 25.1, [Ca] = 0 .

Detectable levels of sodium were present within the basal infoldings (294.2 ± 18.4 mmol Kg^{-1} d.w.), the basal cytoplasm (237.1 ± 16.7 mmol Kg^{-1} d.w.) and the central cytoplasm (172.4 ± 16.0 mmol Kg^{-1} d.w.). However, in the apical part of the tubule cell, the concentration dropped below the element's detection limit. A drop in the concentration of Na was observed from the basal infoldings to the central cytoplasm. The basal cytoplasm Na concentration was lower than that seen in the basal infoldings ($t=2.119$, $P<0.05$), and the central cytoplasm mean concentration was significantly less than the basal cytoplasm value ($t=2.499$, $P<0.05$).

Chloride exhibited no gradient in its distribution. Though the basal cytoplasm Cl concentration (109.9 ± 10.2 mmol Kg^{-1} d.w.) was significantly lower than that of the basal infoldings (152.3 ± 11.3 mmol Kg^{-1} d.w.), t-tests show that a statistically significant pattern was not present within the remainder of the data.

Phosphorus exhibited the highest intracellular concentration of any element analysed. The P concentration in the central cytoplasm was 613.5 ± 17.8 mmol Kg^{-1} d.w. Its distribution within the cells was relatively uniform, as the differences between the concentrations at the basal infoldings and the basal cytoplasm, and the apical cytoplasm and apical microvilli, were not significantly different ($t=0.838$, $P>0.05$ and $t=1.312$, $P>0.05$ respectively). The central cytoplasmic value differed significantly from the basal and apical cytoplasm P concentrations ($t=2.732$, $P<0.05$ and $t=2.575$, $P<0.05$).

The intracellular calcium concentrations were consistently low at all sites and demonstrated no obvious pattern in their distribution. The levels of magnesium in the cell were also constant between each area. Intracellular Mg concentrations varied from approximately 49 to 114 mmol Kg^{-1} d.w. No significant difference was observed between any of the Mg concentrations at adjacent intracellular sites (basal to apical; $t=0.131$; $t=0.029$; $t=0.519$; $t=0.244$; $P>0.05$ in all cases). Sulphur concentrations were also low, ranging from approximately 43 to 106 mmol Kg^{-1} d.w. Both the basal infoldings and apical microvilli had sulphur concentrations that were significantly higher than the adjacent cytoplasm values ($t=3.694$, $P<0.05$ and $t=3.145$, $P<0.05$ respectively). The basal and central cytoplasm concentrations did not differ ($t=1.449$, $P>0.05$), though the central cytoplasmic concentration was higher than the apical concentrations ($t=3.226$, $P<0.05$).

In earlier studies on insect epithelia, the elemental concentrations of Na, K and Cl measured by X-ray microanalysis were considered to represent the ionic form of these elements, fully dissolved in the cytoplasm (Gupta *et al.* 1978; Dow *et al.* 1981; Pivovarova *et al.* 1994a). If the normal dry mass of the cell is taken to be 25% of the wet weight (Dow *et al.* 1981; Pivovarova *et al.* 1994a), then the concentration data for these elements in table 4.4 can be converted from mmol Kg^{-1} dry weight to mmols l^{-1}. Table 4.5 show these estimated values.

Table 4.5. Estimated concentrations of selected elements in mmols l^{-1} in *Locusta* Malpighian tubule cells incubated in control saline for 30 minutes. Data derived from table 4.4. * denotes elemental measurement below the detection limit for that element.

Cell site	*n*	Elemental concentration (mmols l^{-1})		
		K	Na	Cl
Basal infoldings	84	64.3 ± 4.1	98.1 ± 6.1	50.9 ± 3.8
Basal cytoplasm	74	85.7 ± 8.4	79.0 ± 5.6	36.6 ± 3.4
Central cytoplasm	129	115.9 ± 3.7	57.5 ± 5.3	28.8 ± 2.8
Apical cytoplasm	24	159.4 ± 10.9	*	42.7 ± 5.8
Apical microvilli	33	160.3 ± 12.0	*	55.5 ± 9.0

4.3.3. Inhibitors of fluid production.

The effect of 1 mM furosemide (in 0.1% DMSO) on intracellular elemental composition.

Table 4.6 shows the effect of the inhibitor furosemide on the intracellular concentration of the elements in *Locusta* Malpighian tubule cells. Furosemide caused considerable changes in the concentrations of the majority of elements in the tubule cells analysed. Comparison of the basement membrane Na, K and Cl concentrations with those of control saline-incubated tubules indicated that K and

Table 4.6. The elemental concentrations in *Locusta* Malpighian tubules cells after incubation in control saline plus 1 mM furosemide (in 0.1% DMSO) for 30 minutes. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean. ‡ denotes a value significantly different from the appropriate control concentration ($P<0.05$).

		Elemental concentration (mmol Kg^{-1} dry weight)						
Cell site	*n*	K	Na	Cl	P	S	Mg	Ca
Basement membrane	77	133.0 ± 8.7	528.7 ± 20.1	541.7 ± 22.2	83.3 ± 6.8	223.9 ± 15.8	75.5 ± 5.8	33.9 ± 1.7
Basal infoldings	47	144.8 ± 16.9‡	476.0 ± 27.4‡	361.0 ± 24.7‡	302.6 ± 34.8‡	170.6 ± 11.7‡	84.4 ± 7.1	20.8 ± 2.3
Basal cytoplasm	35	195.4 ± 20.3‡	421.0 ± 46.9‡	214.6 ± 25.6‡	586.6 ± 26.8	113.5 ± 9.0	124.1 ± 15.0‡	23.2 ± 7.2
Central cytoplasm	63	126.6 ± 11.3‡	412.0 ± 35.3‡	207.5 ± 17.5‡	540.5 ± 18.6‡	97.9 ± 6.9	114.1 ± 9.7‡	23.3 ± 5.0
Apical cytoplasm	13	241.2 ± 14.1‡	228.0 ± 32.7‡	106.3 ± 5.0	542.9 ± 17.5‡	84.6 ± 9.4	69.6 ± 16.2	*
Apical microvillar border	14	274.0 ± 14.8‡	206.5 ± 19.4‡	78.7 ± 17.8‡	405.1 ± 33.7‡	80.0 ± 6.6	77.3 ± 15.2	*

Cl concentrations were significantly greater in furosemide-treated tubule cells (Mann-Whitney U tests, $P<0.05$ in both cases). No change was observed in the concentration of Na.

The K concentration within the cell was significantly less than in tubule cells incubated in control saline, at all cell sites (basal to apical; $t=2.298$, $P<0.05$; $t=2.623$, $P<0.05$; $t=13.97$, $P<0.001$; $t=6.631$, $P<0.001$; $t=5.305$, $P<0.001$). The gradient in the concentration of K from basal to the apical surface was abolished. The basal and apical cytoplasmic K concentrations (195.4 ± 20.3 and 241.2 ± 14.1 mmol Kg^{-1} d.w. respectively) were not significantly different from the basal infolding and apical microvillar K concentrations (144.8 ± 16.9 mmol Kg^{-1} d.w. and 274.0 ± 14.8 mmol Kg^{-1} d.w. respectively) ($t=1.927$, $P>0.05$ and $t=1.605$, $P>0.05$). The K concentration in the central cytoplasm (126.6 ± 11.3 mmol Kg^{-1} d.w.) was in fact lower then the concentrations in the other 2 areas of the cytoplasm ($t=2.961$, $P<0.05$ and $t=6.340$, $P<0.001$). Clearly, no gradient in this element's concentration was retained in furosemide incubated cells.

Conversely, the Na concentration was raised by incubation in furosemide-containing saline. Each cellular region displayed a higher concentration than that of the control cells (basal to apical; $t=5.677$, $P<0.001$; $t=3.618$, $P<0.001$; $t=6.186$, $P<0.001$; $t=6.631$, $P<0.001$; $t=2.272$, $P<0.05$). However, no clear gradient was present, as the mean values in the upper three regions of the cells were not significantly different from each other ($t=1.007$, $P>0.05$ and $t=0.157$, $P>0.05$). Though the Na concentration in the apical cytoplasm was lower than in the central cytoplasmic zone ($t=3.825$, $P<0.001$), the apical cytoplasmic concentrations was not significantly different from the apical microvillar border value ($t=0.565$, $P>0.05$). The Na concentration was between approximately 476 and 412 mmol Kg^{-1} d.w. in the basal and central cytoplasm region of the cell, and 228 to 207 mmol Kg^{-1} d.w. at the apical cytoplasm and apical microvilli.

An increase in the chloride concentration in furosemide-treated cells was noted. [Cl] was higher than the control tissue in every area except the apical cytoplasm (basal to apical; $t=7.664$, $P<0.001$; $t=3.683$, $P<0.001$; $t=6.219$, $P<0.001$; $t=2.714$, $P<0.05$). A clear pattern in the distribution of this element was not readily apparent though the basal infoldings (361.0 ± 24.7 mmol Kg^{-1} d.w.) possessed a higher concentration of Cl than the neighbouring basal cytoplasm (214.6 ± 25.6 mmol Kg^{-1} d.w.) ($t=4.050$, $P<0.001$). The basal and central cytoplasm concentrations were the same ($t=0.234$, $P>0.05$), as were the apical

cytoplasm and apical microvillar border concentrations (t=1.490, P>0.05). The central cytoplasm concentration was greater than the apical value (t=5.561, P<0.001). The central cytoplasmic concentration of Cl was 270.5 ± 17.5 mmol Kg^{-1} d.w., but the apical microvillar concentration was 78.7 ± 17.8 mmol Kg^{-1} d.w. These data may reflect a slight gradient in the distribution of Cl, decreasing in concentration as analysis moves from the basal to apical surfaces.

Like the control cells, Ca concentrations were correspondingly low intracellularly, and apically were below the detection limit. No pattern in the distribution of this element was observed. The concentration of both Mg and S was somewhat greater at the basal and central areas of furosemide incubated cells. The intracellular phosphorus concentrations at all the analysed sites except the basal cytoplasm (t=1.507, P>0.05) were significantly lower than the control concentrations (from basal and apical to apical surfaces; t=4.611, P<0.001; t=2.843, P<0.05; t=3.978, P<0.001; t=3.857, P<0.001). Within the cytoplasm of the cells, the P concentration was uniform, that is, neither central nor apical cytoplasm concentrations differed significantly from their neighbouring region (t=1.466, P>0.05 and t=0.095, P>0.05 respectively). The P concentrations at both the basal infoldings and the apical microvillar border were both significantly less than at the adjacent cytoplasmic region (t=6.463, P<0.001 and t=3.629, P<0.05 respectively).

Table 4.7 provides elemental concentration data from cells incubated in saline containing 0.1% DMSO alone. This control treatment was performed to investigate the effect of this solvent on cellular elemental distribution, since it was included as a solvent in the stock solution used to make up the furosemide-containing saline. The only element which displayed a consistent difference in its concentration in these cells, in comparison with the control data in Table 4.6, was chloride. In all regions of the cytoplasm, and at the apical microvillar border, the Cl concentration was significantly lower, and was uniform. Central cytoplasmic [Cl] was 29.1 ± 5.3 mmol Kg^{-1}, and excluding the basal infoldings, the other intracellular Cl concentrations were between approximately 27 and 70 mmol Kg^{-1}. None of the three cytoplasm areas Cl concentrations were significantly different from their adjacent area (t=1.891, P>0.05 and t=0.419, P>0.05). The apical cytoplasm and microvillar Cl concentrations were equivalent (t=0.940, P>0.05). Aside from chloride, the remaining elements were present at concentrations which did not significantly differ from Malpighian tubule cells incubated in control

Table 4.7. The elemental concentrations in *Locusta* Malpighian tubules cells after incubation in control saline plus 0.1% DMSO for 30 minutes. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean. * denotes elemental measurement below the detection limit for that element. ‡ denotes a value significantly different from the appropriate control concentration ($P<0.05$).

		Elemental concentration (mmol Kg^{-1} dry weight)						
Cell site	*n*	K	Na	Cl	P	S	Mg	Ca
Basement membrane	26	94.7 ± 4.7	520.5 ±18.5	427.7 ± 12.4	68.3 ± 11.6	151.2 ± 9.8	78.9 ± 7.3	31.9 ± 4.0
Basal infoldings	46	193.1 ± 10.4	401.1 ± 25.8‡	200.3 ± 22.7	448.4 ± 21.9	88.7 ± 6.9	62.1 ± 9.0	35.1 ± 5.5
Basal cytoplasm	30	275.1 ± 19.2	246.5 ± 24.6	70.1 ± 7.8‡	625.8 ± 31.6‡	84.3 ± 9.6‡	72.0 ± 9.1	14.9 ± 4.2
Central cytoplasm	20	456.1 ± 30.1	141.5 ± 20.9	29.1 ± 5.3‡	762.4 ± 26.2	69.6 ± 9.1	73.5 ± 9.1	*
Apical cytoplasm	17	554.1 ± 22.3	*	47.0 ± 12.2‡	696.0 ± 57.2	64.5 ± 6.0	63.8 ± 13.9	24.4 ± 8.5
Apical microvillar border	6	543.0 ± 70.7	*	26.6 ± 16.4‡	739.9 ± 67.6	37.1 ± 16.9‡	110.7 ± 45.6	*

saline at the majority of sites. The P concentration in the DMSO-treated tissue was, at all but one of the measured intracellular sites, the same as that of control saline-treated cells. The S and Mg concentrations intracellularly were also of similar magnitudes in most zones of the cell.

The effect of 1μM bafilomycin A_1 on intracellular elemental composition.

Table 4.8 contains the intracellular elemental concentrations of tubules cells incubated in saline containing 1μM bafilomycin A_1. At the basement membrane, statistical analysis indicated that the K concentration was significantly higher than in control-saline incubated tubule cells (Mann-Whitney U-test, $P<0.05$), but that no change occurred in the concentrations of Na or Cl.

The intracellular concentration of K increased in the basal region of the cell, as both the basal infoldings ($t=4.756$, $P<0.001$) and the basal cytoplasm ($t=5.556$, $P<0.001$) concentration were higher than the control values. The former concentration was 346.4 ± 26.2 mmol Kg^{-1} d.w., whilst the latter was 423.5 ± 26.3 mmol Kg^{-1} d.w. Whilst the central and apical cytoplasm concentrations were not significantly different from the control readings ($t=1.302$, $P>0.05$ and $t=0.095$, $P>0.05$ respectively), at the apical microvillar border, the concentration of K (350.0 ± 15.2 mmol Kg^{-1} d.w.) was lower ($t=3.340$, $P<0.05$). The central and apical cytoplasm K concentrations were approximately 384 and 472 mmol Kg^{-1} d.w. respectively. None of the cytoplasmic K values were significantly different from each other within the bafilomycin A_1 data set ($t=0.862$, $P>0.05$ and $t=1.433$, $P>0.05$). The basal infoldings K concentration was lower than that of the basal cytoplasm ($t=2.055$, $P<0.05$), as was the apical microvillar concentration in comparison with the apical cytoplasm ($t=2.443$, $P<0.05$). The statistical tests within the bafilomycin data set suggests that a gradient of K was no longer present within the cytoplasm.

The intracellular concentration of Na was below its detection limit throughout the cytoplasm and the apical microvillar border, though the basal infolding concentration, 255.3 ± 32.3 mmol Kg^{-1} d.w., was not significantly different from that of the control cells ($t=0.866$, $P>0.05$).

The chloride concentration at all the cell sites measured, except the apical microvillar border, was significantly greater than the control values (from basal infoldings to apical cytoplasm; $t=5.338$, $P<0.001$; $t=8.532$, $P<0.001$;

Table 4.8. The elemental concentrations in *Locusta* Malpighian tubules cells after incubation in control saline plus 1μM bafilomycin A_1. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean. * denotes elemental measurement below the detection limit for that element. ‡ denotes a value significantly different from the appropriate control concentration ($P<0.05$).

		Elemental concentration (mmol Kg^{-1} dry weight)						
Cell site	*n*	K	Na	Cl	P	S	Mg	Ca
Basement membrane	18	135.5 ± 16.9	419.5 ± 43.9	546.7 ± 53.6	56.4 ± 9.6	270.7 ± 20.3	110.8 ± 20.4	44.1 ± 4.7
Basal infoldings	14	346.4 ± 26.2‡	255.3 ± 32.3	307.7 ± 20.4‡	630.0 ± 48.3	173.9 ± 14.8‡	85.4 ± 13.3	*
Basal cytoplasm	17	423.5 ± 26.3‡	*	274.5 ± 14.8‡	674.8 ± 38.2‡	117.6 ± 7.8‡	73.6 ± 12.9	18.4 ± 7.0
Central cytoplasm	14	383.8 ± 38.5	*	209.6 ± 13.0‡	688.4 ± 38.2	98.0 ± 7.5‡	68.2 ± 14.5	*
Apical cytoplasm	10	472.4 ± 47.7	*	227.2 ± 10.3‡	710.9 ± 60.2	78.1 ± 6.1‡	42.2 ± 13.3	*
Apical microvillar border	9	350.0 ± 15.2‡	*	200.1 ± 26.4	425.9 ± 10.2‡	130.6 ± 7.7	42.2 ± 8.0‡	*

t=7.727, P<0.001; t=4.876, P<0.05). The Cl concentration at the basal infoldings of the bafilomycin A_1 treated cells was not statistically significantly different from the basal cytoplasm value (t=1.347, P>0.05), though it was higher than that recorded from the central cytoplasm (t=3.145, P<0.05). The Cl concentration at the other intracellular regions did not significantly differ from each other, hence, no gradient was present. The central cytoplasm Cl concentration was approximately 210 mmol Kg^{-1} d.w.

Phosphorus concentrations differed from those seen in control saline treated tubule cells in only 2 of 5 intracellular sites. At the apical microvillar border, the P concentration was less than that seen in control treated cells (t=4.144, P<0.05), but in the basal cytoplasm, it was greater (t=2.660, P<0.05). Bafilomycin-treated cells exhibited Ca concentrations below the detection limit in the majority of intracellular sites, the sole exception being the basal cytoplasm. Additionally, the Mg concentration only significantly differed from the control tissue at one site, the apical microvilli (t=3.202, P<0.05). In contrast, the intracellular S concentrations were all significantly different from the controls, though the cytoplasm concentrations of this element were not significantly different from each other (t=1.753, P>0.05 and t=1.921, P>0.05).

The effect of replacing K with Rb on intracellular elemental composition.

The effect on intracellular elemental distribution when 8.6 mM [K^+] was replaced with 8.6 mM [Rb^+] in the bathing saline is presented in Table 4.9. Figure 4.5 shows a series of typical spectra recorded from areas of cells incubated in Rb-containing saline. Only a small number of readings from the basal and apical cytoplasmic sites could be made with confidence in the exact location of analysis (n = 7 and n = 4 respectively). Values from these areas are therefore excluded. Na and Cl concentrations were again highest in the basement membrane region, and as might be expected, a noticeable decrease in the concentration of K occurred. Furthermore, though the control saline-incubated basement membrane K concentration was approximately 98 mmol Kg^{-1} d.w., the corresponding Rb-saline concentrations was only 20 mmol Kg^{-1} d.w.

Predictably, the intracellular K concentration also declined in all areas of the cell, with all the readings being significantly different from the control values.

Table 4.9. The elemental concentrations in *Locusta* Malpighian tubules cells after incubation in saline containing 8.6 mM Rb. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean. * denotes elemental measurement below the detection limit for that element. ‡ denotes a value significantly different from the appropriate control concentration ($P<0.05$). n values are, basement membrane =17; basal infoldings = 13; central cytoplasm = 18; apical microvillar border = 34.

	Elemental concentrations (mmol Kg^{-1} dry weight)							
Cell site	K	Rb	Na	Cl	P	S	Mg	Ca
Basement membrane	19.8±3.8	85.3 ± 5.5	416.3 ± 31.8	438.8 ± 34.6	54.6 ± 7.3	217.2 ± 31.8	102.6 ± 18.7	33.6 ± 4.2
Basal infoldings	47.4 ± 6.6‡	137.6 ± 8.9	218.9 ± 37.9	43.9 ± 7.3‡	442.0 ± 35.3	67.8 ± 8.4‡	78.2 ± 17.3	*
Basal cytoplasm	–	–	–	–	–	–	–	–
Central cytoplasm	77.1 ± 4.8‡	152.9 ± 5.0	*	33.8 ± 5.3‡	564.5 ± 16.3	77.1 ± 4.8	56.1 ± 10.5‡	*
Apical cytoplasm	–	–	–	–	–	–	–	–
Apical microvillar border	97.4 ± 6.7‡	192.5 ± 16.3	193.0 ± 40.8	69.3 ± 6.3‡	608.5 ± 40.1	97.4 ± 6.7	88.3 ± 11.1	*

Figure 4.5. Five typical spectra recorded from Malpighian tubule cells incubated in Rb-containing saline. BMV137, AMV 116 are abbreviations referring to the area from which the spectra was recorded. Vertical axis represents total counts, horizontal axis represents energy channels (keV). The scale of the vertical axis varies.

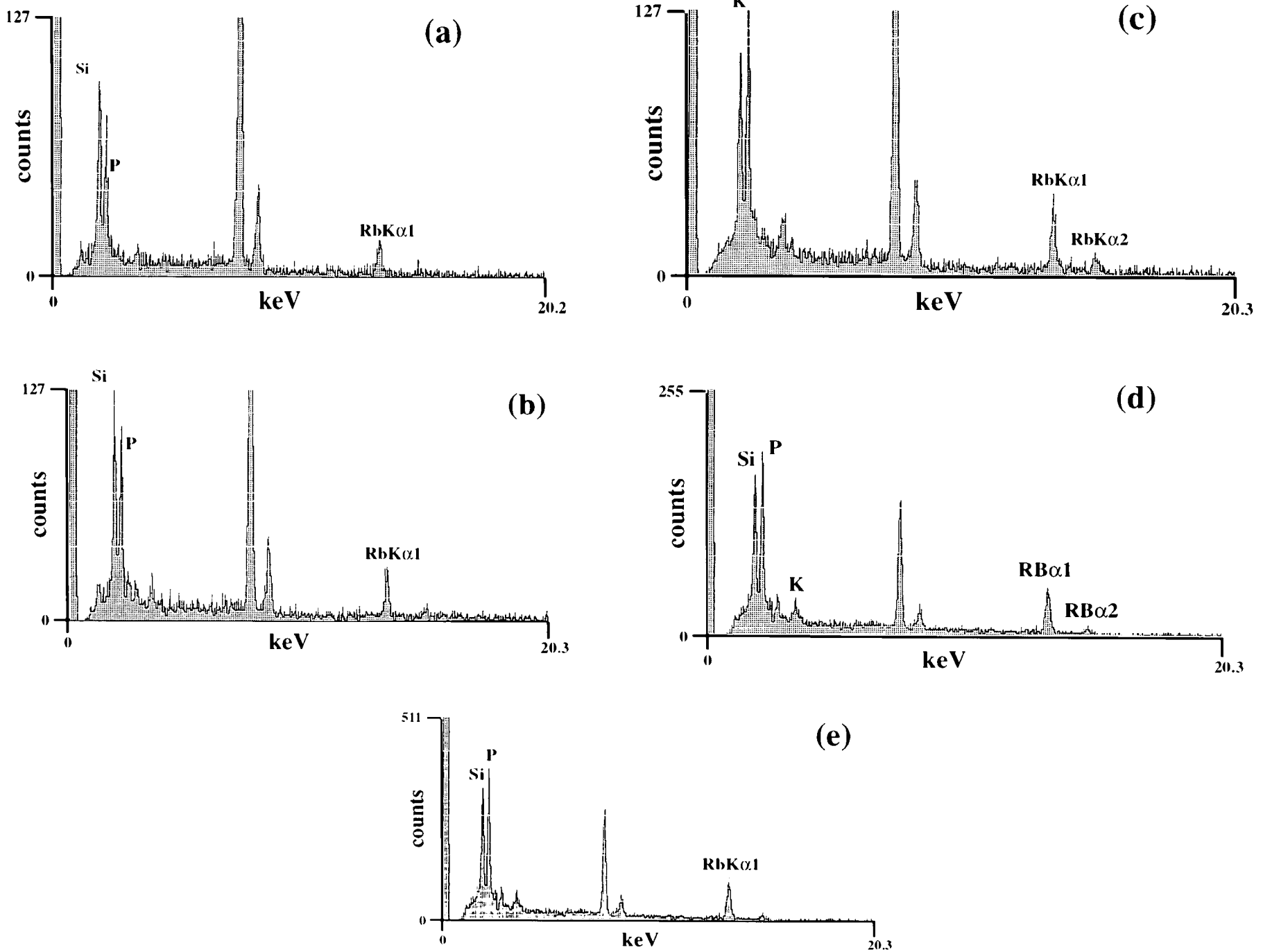
(a). **BMV 137**. Peaks (left to right), Si Kα, P Kα, Ni Kα, Ni Kβ, Rb Kα1. Elemental concentrations were; [Na] = 290.3, [K] = 46.5, [P] = 435.7, [S] = 18.7, [Cl] = 0, [Mg] = 118.7, [Ca] = 6.6, [Rb] = 160.0 .

(b). **CYT 305** (basal cytoplasm). Peaks (left to right), Na Kα, Si Kα, P Kα, Ni Kα, Ni Kβ and Rb Kα1. Elemental concentrations were; [Na] = 162.9, [K] = 34.9, [P] = 526.0, [S] = 74.6, [Cl] = 51.0, [Mg] = 0, [Ca] = 26.6, [Rb] = 171.9 .

(c). **CYT 309** (central cytoplasm). Peaks (left to right), Si Kα, P Kα, K Kα, Ni Kα, Ni Kβ, Rb Kα1 and Rb Kβ1. Elemental concentrations were; [Na] = 0, [K] = 57.4, [P] = 562.2, [S] = 62.0, [Cl] = 27.4, [Mg] = 65.0, [Ca] = 17.4, [Rb] = 158.2 .

(d). **CYT 319** (apical cytoplasm). Peaks (left to right), Si Kα, P Kα, K Kα, Ni Kα, Ni Kβ, Rb Kα1 and Rb Kβ1. Elemental concentrations were; [Na] = 102.7, [K] = 90.7, [P] = 590.4, [S] = 54.8, [Cl] = 42.3, [Mg] = 108.6, [Ca] = 16.4, [Rb] = 146.4 .

(e). **AMV 116**. Peaks (left to right), Si Kα. P Kα, Cl Kα, K Kα, Ni Kα, Ni Kβ and Rb Kα1. Elemental concentrations were; [Na] = 0, [K] = 59.9, [P] = 658.3, [S] = 55.3, [Cl] = 76.9, [Mg] = 41.5, [Ca] = 0, [Rb] = 209.5 .


(a)
127
counts
0
Si
P
RbKα1
0
keV
20.2
(c)
127
counts
0
K
RbKα1
RbKα2
0
keV
20.3
(b)
127
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0
Si
P
RbKα1
0
keV
20.3
(d)
255
counts
0
Si
P
K
RBα1
RBα2
0
keV
20.3
(e)
511
counts
0
P
Si
RbKα1
0
keV
20.3

For example, the central cytoplasmic K concentration was 77.1 ± 4.8 mmol Kg^{-1} dry weight, significantly lower than the control value of approximately 348 mmol Kg^{-1} d.w. The results suggest a concentration gradient of K was retained from the basal to apical surfaces, and statistical analysis indicates that the basal microvilli concentration was significantly less than the mean concentration in the central cytoplasm region ($t=3.370$, $P<0.05$). Similarly, the central cytoplasmic concentration of K was lower than that of the apical microvillar border ($t=2.477$, $P<0.05$).

The Rb concentration does not exhibit such a clear gradient, as the basal infoldings mean Rb concentration was not significantly different from that of the central cytoplasm ($t=1.601$, $P>0.05$). The basal infoldings Rb concentration was 137.6 ± 8.9 mmol Kg^{-1} d.w., and the central cytoplasm concentration was 152.9 ± 50 mmol Kg^{-1} d.w. However, there was a significantly greater Rb concentration at the apical microvillar border than in the central cytoplasm ($t=2.31$, $P<0.05$). This indicates that the usual K concentration gradient may have been replaced to a limited extent by Rb, though it is clear that Rb concentrations intracellularly are much lower than the usual levels recorded for K in control cells.

The Na concentration in the Rb-saline incubated cells was not significantly different from control tissue in the basal infolding region ($t=1.533$, $P>0.05$), though the apical microvilli mean concentration (192.5 ± 40.8 mmol Kg^{-1}) was above the detection limit, unlike the same site in control saline-incubated tissue.

The Cl concentrations within the cell displayed a central cytoplasm concentration lower than that seen in the apical microvillar border ($t=4.416$, $P<0.05$). The mean Cl concentrations at the basal infoldings and the central cytoplasm were not significantly different. Nevertheless, the mean Cl concentrations were substantially less in all the measured sites in comparison with the control treated tubule cells. The central cytoplasm Cl concentration was 33.8 ± 5.3 mmol Kg^{-1} d.w., whilst the concentration at the apical microvillar border was 69.3 ± 6.3 mmol Kg^{-1} d.w. Again, a partial gradient in the concentration of this element may have been present.

The intracellular P concentration was unchanged when compared with control Malpighian tubule cells. The concentration of S differed also only at one intracellular site, the basal infoldings ($t=2.136$, $P<0.05$). Mg differed only in the

central cytoplasm (t=5.302, P<0.001). All the areas of the cell displayed mean Ca concentrations below that of the detection limit for this element.

4.3.4. Stimulators of fluid production.

The effect of 1 mM DB-cAMP on intracellular elemental composition.

Table 4.10 gives the elemental content of Malpighian tubule cells incubated in control saline containing the fluid secretion stimulator 1 mM DB-cAMP. The intracellular differences between these cells and those incubated in control saline were confined to K, Na and Cl. Basement membrane elemental concentrations followed the previously observed pattern. No change was noted in the concentrations of Na, K, and Cl in comparison with control basement membrane values (Mann-Whitney U-tests, P>0.05 in all cases).

Intracellular K was lower than the control tissue values (basal to apical; t=4.411, P<0.001; t=2.329, P<0.05; t=2.736, P<0.05; t=3.299, P<0.05; t=3.582, P<0.001), but in contrast to effect of inhibitors, a gradient of concentration running from the basal to apical membranes was retained. The intracellular K concentrations ranged from 130.5 ± 6.7 mmol Kg^{-1} d.w. at the basal infoldings, to 261.3 ± 29.5 mmol Kg^{-1} d.w. in the central cytoplasm, reaching a maximum in the apical region of the cell of approximately 340 mmol Kg^{-1} d.w. The basal infolding concentration was lower than that in the basal cytoplasm (t=5.672, P<0.001). The central cytoplasmic K concentration was not significantly greater the basal cytoplasm measurement (t=1.467, P>0.05). The control central cytoplasmic K concentration was smaller than the apical cytoplasm K concentration (t=2.127, P<0.05). In a repeat of the pattern observed in control incubated tubule cells, the apical cytoplasm concentration did not differ from the apical microvillar value (t=0.154, P>0.05). It is clear that a concentration gradient of K still occurs in these stimulated cells.

Na levels within the cell were altered by stimulation with DB-cAMP. Both the basal infoldings and the basal cytoplasm displayed lower concentrations than recorded from control cells (t=3.519, P<0.001 and t=3.734, P<0.001 respectively), whilst the central cytoplasm value was not significantly different from the appropriate control (t=0.068, P>0.05). Apically, both the apical cytoplasm and apical microvilli exhibited mean concentrations above the

Table 4.10. The elemental concentrations in *Locusta* Malpighian tubules cells after incubation in control saline plus 1mM DB-cAMP. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean. * denotes elemental measurement below the detection limit for that element. ‡ denotes a value significantly different from the appropriate control concentration ($P<0.05$).

		Elemental concentration (mmol Kg^{-1} dry weight)						
Cell site	*n*	K	Na	Cl	P	S	Mg	Ca
Basement membrane	26	105.3 ± 6.9	558.0 ± 36.1	509.1 ± 30.4	200.7 ± 23.3	154.1 ± 11.2	68.1 ± 5.6	17.6 ± 1.6
Basal infoldings	14	130.5 ± 6.7‡	205.4 ± 17.3‡	206.0 ± 25.9‡	357.4 ± 18.2	56.5 ± 5.3	48.5 ± 8.9	*
Basal cytoplasm	17	214.4 ± 12.9‡	146.2 ± 14.3‡	185.5 ± 13.9‡	532.9 ± 31.0	43.4 ± 5.2	67.7 ± 8.4	*
Central cytoplasm	37	261.6 ± 29.5‡	174.2 ± 20.6	203.6 ± 25.4‡	656.9 ± 57.1	88.9 ± 24.7	113.7 ± 15.9	*
Apical cytoplasm	12	343.0 ±24.4‡	143.5 ± 15.5	391.7 ± 19.7‡	695.8 ± 32.1	105.6 ± 8.3	68.8 ± 9.0	*
Apical microvillar border	21	338.6 ± 16.5‡	174.0 ± 16.4	368.5 ± 16.6‡	553.9 ± 33.4	78.1 ± 4.5	78.4 ± 7.1	*

detection limit for Na. However, only the basal infoldings and the basal cytoplasm mean Na concentrations were significantly different from each other, the latter being lower ($t=2.670$, $P<0.05$). The rest of the Na concentrations were not significantly different from each other (basal to apical; $t=1.117$; $t=1.189$; $t=1.236$; in all cases $P>0.05$). This suggests that a higher overall [Na] was accompanied by a relatively constant intracellular concentration.

DB-cAMP-stimulated cells had higher concentration of chloride than control cells at all the measured cell sites (basal to apical; $t=2.596$, $P<0.05$; $t=4.060$, $P<0.001$; $t=4.301$, $P<0.001$; $t=9.263$, $P<0.001$; $t=6.364$, $P<0.001$). The Cl concentrations rose as high as 391.7 ± 19.7 mmol Kg^{-1} d.w. at the apical cytoplasm, almost 2½ times greater than corresponding control concentration. In the basal region of the DB-cAMP stimulated cells, and in the central cytoplasm, the mean Cl concentration was approximately 200 mmol Kg^{-1} d.w. Nevertheless, the concentrations in the basal infoldings and the basal and central cytoplasm were not significantly different from their neighbouring intracellular region ($t=0.940$, $P>0.05$; $t=0.616$, $P>0.05$). The apical cytoplasm concentration was significantly greater than the central Cl concentration ($t=5.784$, $P<0.05$), but the difference between the concentrations at the apical cytoplasm and the apical microvillar border did not reach statistical significance ($t=0.871$, $P>0.05$). These data suggest a rise in [Cl] but without a noticeable gradient in concentration present intracellularly.

The elemental concentrations of P, S and Mg were not significantly different from appropriate control values at any of the measured regions of the DB-cAMP stimulated tubule cells. Throughout the measured cellular sites, the mean Ca concentrations were below the detection limit of this element.

4.3.7. The effect of corpora cardiaca on intracellular elemental concentration.

The elemental concentrations in tubule cells stimulated with corpora cardiaca extract at a concentration of 0.1 gland pairs per ml of control saline is given in Table 4.11. The basement membrane of these stimulated cells exhibited a similar pattern in elemental distribution to control cells, with Na and Cl being the elements present in the highest concentration at this site. The basement membrane mean elemental concentrations of Na, K, and Cl were all significantly

Table 4.11. The elemental concentrations in *Locusta* Malpighian tubules cells after incubation in control saline plus 0.1 gland pairs per ml of saline corpora cardiaca extract. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean. * denotes elemental measurement below the detection limit for that element. ‡ denotes a value significantly different from the appropriate control concentration ($P<0.05$).

		Elemental concentration (mmol Kg^{-1} dry weight)						
Cell site	*n*	K	Na	Cl	P	S	Mg	Ca
Basement membrane	33	163.0 ± 7.5	657.8 ± 26.1	556.4 ± 19.6	84.3 ±11.6	280.2 ± 12.1	115.2 ± 9.4	51.6 ± 2.6
Basal infoldings	32	235.2 ± 9.7‡	497.9 ± 31.6‡	249.0 ± 16.4‡	665.1 ± 23.6‡	96.6 ± 5.1	114.3 ± 10.6	12.1 ± 2.0
Basal cytoplasm	15	264.5 ± 9.9	129.7 ± 21.5‡	111.3 ± 6.8	681.6 ± 33.4‡	69.0 ±6.1	73.6 ± 7.3	*
Central cytoplasm	19	320.1 ± 18.7	*	118.1 ± 7.8‡	709.9 ± 46.4	69.4 ± 6.4	78.0 ± 8.6	*
Apical cytoplasm	9	406.4 ± 11.4‡	164.8 ± 19.0	118.0 ± 9.2	836.0 ± 65.4	72.3 ± 9.3‡	104.4 ± 1.6	*
Apical microvillar border	11	530.9 ± 51.8	190.0 ± 35.9	223.2 ± 28.0	758.1 ± 66.3	65.4 ± 7.7	177.6 ± 20.0‡	*

greater than control cell basement membrane concentrations (Mann-Whitney U-tests, $P<0.05$ in all cases).

Within the corpora cardiaca-stimulated cells, the basal region displayed the largest number of differences in comparison with the control tissue. Mean K concentrations were significantly different from tubule cells incubated in control saline at two sites, namely greater at the basal infoldings ($t=2.694$, $P<0.05$) and the lower at the apical cytoplasm ($t=2.062$, $P<0.05$). The former K concentration was 235.2 ± 9.7 mmol Kg^{-1} d.w., the latter, 406.4 ± 11.4 mmol Kg^{-1} d.w. A gradient of concentration was maintained, from the basal cytoplasm to the apical microvilli, of similar magnitude to that seen in control cells. The central cytoplasmic value was 320.1 ± 18.7 mmol Kg^{-1} d.w., and the apical microvillar border value was 530.9 ± 51.8 mmol Kg^{-1} d.w. Analysis of the corpora cardiaca extract data indicated that only the K concentrations of the basal infoldings and the basal cytoplasm were not significantly different ($t=1.866$, $P>0.05$). The remaining basal-side intracellular concentrations of K were significantly lower than their neighbouring apical site (basal to apical; $t=2.711$; $t=2.272$; $t=2.345$; in all cases $P<0.05$). Essentially, a basal to apical concentration gradient of K was retained in corpora cardiaca stimulated cells.

Corpora cardiaca extract caused changes in the intracellular Na concentrations, though at individual cell sites these changes showed some variability. The basal infoldings concentration of Na was 497.9 ± 31.6 mmol Kg^{-1} d.w. ($t=5.721$, $P<0.001$), but the basal cytoplasm concentration was lower, only 129.7 ± 21.5 mmol Kg^{-1} d.w. ($t=3.682$, $P<0.001$). The Na concentration in the central cytoplasmic zone was below the detection limit, yet in both apical regions the mean concentrations are above the detection limit for Na. The apical cytoplasmic Na concentration was 164.8 ± 19.0 mmol Kg^{-1} d.w., and the apical microvillar border concentration was 190.0 ± 35.9 mmol Kg^{-1} d.w. Though the Na concentration at the basal infolding region is greater than the nearby basal cytoplasm ($t=9.635$, $P<0.001$), no clear pattern could be discerned in the distribution of Na in the apical region, since the concentrations of Na did not significantly differ between the 2 sites ($t=0.622$, $P>0.05$).

Chloride was present in broadly similar mean concentrations within the corpora cardiaca treated tissue as in control incubated tissue, though there were significant differences at two sites, the basal infoldings ($t=4.592$, $P<0.001$) and the central cytoplasm ($t=3.131$, $P<0.05$), where in both cases the concentration

was greater in the corpora cardiaca stimulated cells. The central cytoplasm Cl concentration was 118.1 ± 7.8 mmol Kg^{-1} d.w., and the basal infolding concentration was 249.0 ± 16.4 mmol Kg^{-1} d.w. The cytoplasmic concentration of Cl was uniform within the three subdivisions of the cytoplasm ($t=1.312$, $P>0.05$; $t=0.572$, $P>0.05$). Within the corpora cardiaca data, the basal infoldings Cl concentration was greater than that of the basal cytoplasm ($t=7.772$, $P<0.001$). Similarly, the apical microvillar border mean Cl concentration is greater than at the apical cytoplasm ($t=3.570$, $P<0.05$). This pattern was broadly similar to that seen in control saline-treated tubule cells.

Intracellular P concentrations were significantly higher than control values at the basal infoldings, and the basal and central cytoplasmic regions of the corpora cardiaca extract-treated cells (basal to apical; $t= 4.643$, $P<0.001$; $t=3.622$, $P<0.05$; $t=2.692$, $P<0.05$). No gradient in the concentration of P was observed since none of the mean intracellular values were significantly different from the adjacent value (basal to apical; $t=0.399$; $t=1.110$; $t=1.018$; $t=0.826$; in all cases, $P>0.05$). Mean Ca concentrations were all below this element's detection limit, except at the basal infoldings. Mg concentrations were all similar to mean control concentrations, except at the basal infoldings ($t=2.360$, $P<0.05$) and the apical microvilli ($t=3.939$, $P<0.001$) where the concentrations were greater. S concentrations differed solely at the apical cytoplasm, where it was higher than the appropriate mean control concentration ($t=2.387$, $P<0.05$).

4.3.5. Mass dense concretions.

Both conventionally prepared sections of *Locusta* Malpighian tubules and cryofixed material for quantitative X-ray microanalysis contained mass dense concretions, also known as dark bodies (Pivovarova *et al.* 1994a). Conventionally prepared sections indicated that these structures were clearly different from mitochondria (see Figure 4.3). Dark bodies were seen in relatively small numbers in most cryofixed sections. Resin embedded sections suggested that the dark bodies' were distributed throughout the cytoplasm, and so the small size of the area suitable for microanalysis in most cryofixed sections frequently contained only a few recognisable concretions. Thus, the fragmented nature of the cryofixed sections, and additional difficulties in identifying concretions combined to create conditions in which it was difficult to systematically investigate the

effects on the content of these structures of all the stimulators and inhibitors used in this chapter. Adequate numbers of concretions were observed only in tubule cells stimulated with DB-cAMP and corpora cardiaca extract, though small numbers of concretions were observed in all the experimental treatments. For example, concretions were analysed in tubule cells incubated in Rb-saline, and the earlier finding that these bodies replaced K with Rb confirmed (Pivovarova *et al.* 1994a). However, since control saline-treated tissue was the most extensively investigated treatment, a large number of dark bodies were analysed in this tissue.

Mass dense concretions in Malpighian tubule cells incubated in control saline.

Mass dense concretions were analysed from Malpighian tubule cells incubated for 30 minutes in control saline. As previously reported in this tissue, 3 types of concretions could be discerned in control cells on the basis of their elemental content (Pivovarova *et al.* 1994a). In this earlier study, the 3 types of mass dense bodies were termed A, B and C type concretions, and this convention is followed here. Three representative spectra, one taken from each of these types are shown in Figure 4.6. It was not possible to differentiate between the concretions types in terms of their physical appearance, though the concretions were elliptical or circular in cross-section with a diameter of between approximately 0.3 and 5.0µm. Table 4.12 gives the mean elemental concentration of the 3 types of concentration.

No clear pattern of distribution of the three types of concretions could be elucidated, though they were seemingly confined to the cytoplasm. Conventionally fixed tissue did not reveal the presence of concretions in the tubule lumen, though reports from other species, for example *Drosophila hydei* (Wessing *et al.* 1992), have indicated their presence in this region. The density of the all three types of dark body exceeded that of the surrounding cytoplasm. The appearance of the concretions was very similar in conventionally prepared and cryofixed material (see Figure 4.3).

The most numerous concretion observed in tubules incubated in control saline was the type A. Type A concretion P and Ca concentrations were in the approximate ratio 1:1 (r^2 = 0.841, $P<0.001$). The mean P concentration was

Figure 4.6. Three typical spectra recorded from mass dense concretions present in control saline-incubated *Locusta* Malpighian tubule cells. All units in mmol Kg^{-1} dry weight. DB 129, DB 253 are abbreviations referring to the exact concretion (DB is an abbreviation for dark body).

(a). **A type** (DB129). Elemental concentrations were; [Na] = 193.6, [K] = 204.6, [P] = 2521.9, [S] = 23.5, [Cl] = 44.1, [Mg] = 345.2 and [Ca] = 2211.8 .

(b). **B type** (DB161). Elemental concentrations were; [Na] = 24.4, [K] = 197.3, [P] = 27.2, [S] = 566.9, [Cl] = 90.6, [Mg] = 31.8 and [Ca] = 5.0 .

(c). **C type** (DB253). Elemental concentrations were; [Na] = 38.2, [K] = 418.5, [P] = 19.1, [S] = 80.2, [Cl] = 74.7, [Mg] = 319.9 and [Ca] = 10.1 .

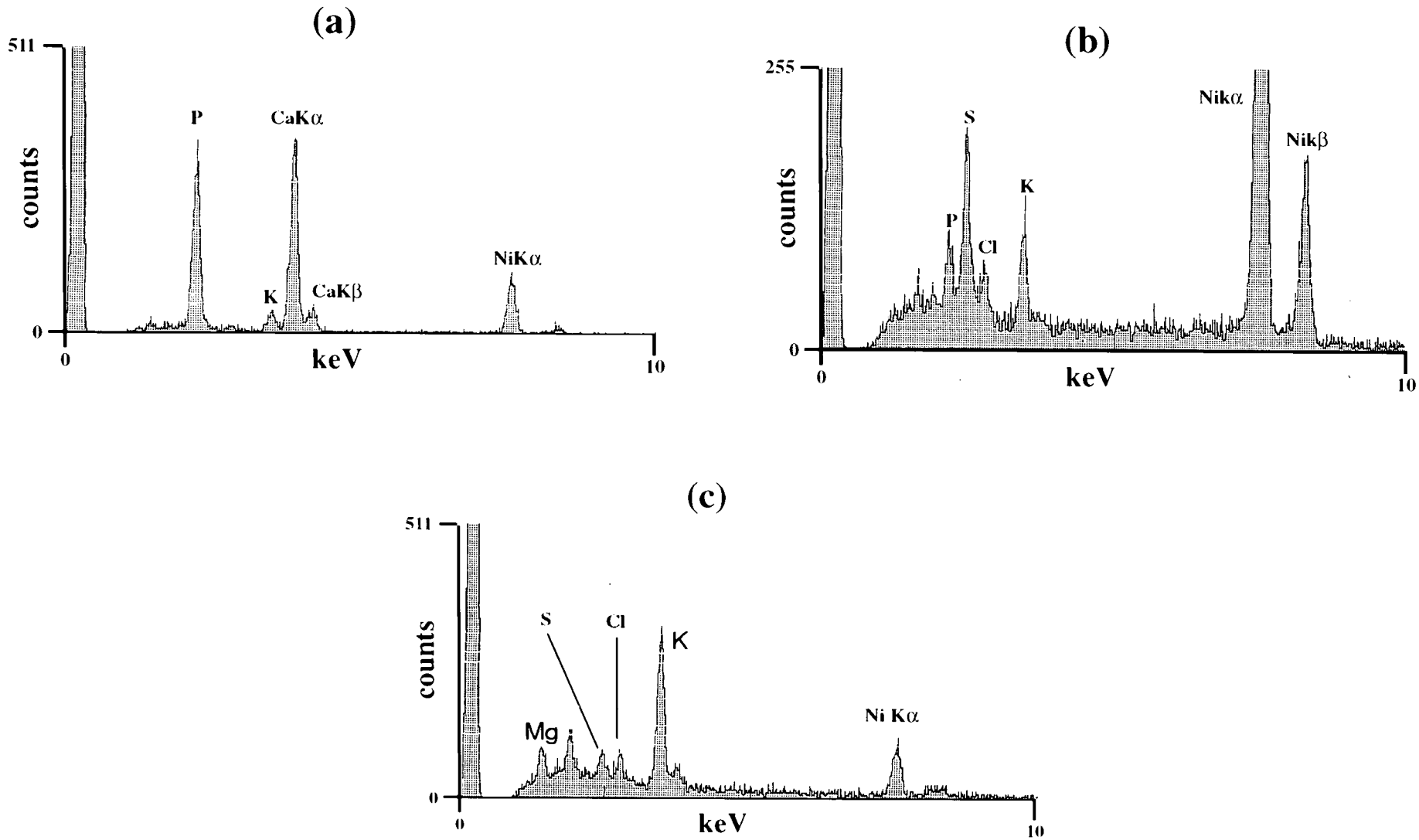
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Table 4.12. The elemental composition and mass density of mass dense concretions in tubules incubated in control saline. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean.

			Elemental concentration (mmol Kg^{-1} dry weight)						
Concretion	Mass density	*n*	K	Na	Cl	P	S	Mg	Ca
A type	2.52	47	241.6 ± 17.4	302.3 ± 72.2	212.7 ± 54.7	1998.8 ± 110.0	116.8 ± 22.0	199.3 ± 19.4	1698.6 ± 105
B type	2.24	21	169.6 ± 13.9	199.0 ± 38.3	133.6 ± 7.6	100.0 ± 16.8	316.6 ± 21.6	47.6 ± 7.1	*
C type	1.59	33	223.3 ± 20.0	204.8 ± 41.0	92.1 ± 23.2	143.9 ± 25.4	91.3 ± 13.8	240.1 ± 16.3	*

approximately 2000 mmol Kg^{-1} d.w., and the mean Ca concentration was slightly lower at approximately 1700 mmol Kg^{-1} d.w. The mean concentrations of P and Ca present in these structures surpassed the concentration of any other element measured in this study with X-ray microanalysis; the maximum concentration recorded was a Type A body with [P] approximately equal to 4450 mmol Kg^{-1} dry weight. Na and Cl were present in a ratio of concentrations of about 1:1 (r^2=0.619, P<0.001). The sodium concentration was 302.3 ± 72.2 mmol Kg^{-1} d.w. and the Cl concentration was 212.7 ± 54.7 mmol Kg^{-1} d.w. An appreciable concentration of K is also present, this concentration was 241.6 ± 17.4 mmol Kg^{-1} d.w., with lesser concentrations of Mg and S present. The mean mass density of this concretion was the highest of the three types measured, being 2.52 times greater than the adjacent cytoplasm. Mean type A concretion concentrations of Na, Cl, S and Mg were higher than that ordinarily observed in the cytoplasm of *Locusta* Malpighian tubules, and taking into account the mass density, it is clear that these concretions contained a substantially greater amount of the measured elements, than would be present in an equivalent area of the cytoplasm. The K concentration was approximately the same as normally seen in the cytoplasm, but the mass density suggests a considerable amount of this element was also present in this concretion type.

Type B concretions differed from type A. The predominant element in terms of mean concentration was clearly S, followed by Na. The S concentration was 316.6 ± 21.6 mmol Kg^{-1} d.w., whilst the Na concentration was 199.0 ± 38.3 mmol Kg^{-1} d.w. There was no statistically significant relationship between the concentration of these two elements in the concretions (r^2 = 0.005, P>0.05). K was the element present at the third highest mean concentration, 169.6 ± 13.9 mmol Kg^{-1} d.w. Type B bodies possessed a negligible Ca content. K, Na and Cl displayed mean concentrations comparable to those usually seen intracellularly in control-treated cells. P, Mg and Ca appeared relatively minor components of type B dark bodies, with concentrations of approximately 100, 134 and 48 mmol Kg^{-1} d.w. in this concretion type. Since the mass density of this concretion type (2.24) was only slightly less than the A type, these electron dense structures also retained significant amounts of the measured elements. Type B concretions were the least common form recorded in control Malpighian tubule cells.

C-type concretions were the least substantial, in terms of mass density, of the three types seen in tubule cells incubated in control saline. Their mean mass

density was 1.59. Mg, K and Na were the three elements present in the highest concentrations in the C type bodies. Mg and K were present at very similar mean concentrations, 240.1 ± 16.3 mmol Kg^{-1} d.w. and 223.3 ± 20.0 mmol Kg^{-1} d.w. respectively, but the distribution of these two elements was not significantly related (r^2 = 0.031, P>0.05). The Na concentration was 204.8 ± 41.0 mmol Kg^{-1} d.w. Again, Ca below its detection limit, and S, Cl and K present at concentrations closely matching the cytoplasmic control values for these elements. The approximate concentrations of S and Cl were 91 and 92 mmol Kg^{-1} d.w. Mg was concentrated in comparison with mean intracellular values, but the mean Na concentration was of the same order as control microanalysis observations at the basal regions. The mean concentrations of S and Cl were similar, and these concentrations are present in an approximate ratio of 1:1 (r^2 = 0.296, P<0.05). The concentration of K in these cells was approximately 233 mmol Kg^{-1} d.w. Though the mass density of these concretions is the lowest of the three types, C-types must still contain considerable amounts of elements, particularly Mg, K and Na.

Stimulators of fluid production.

The effect of 1 mM DB-cAMP on the composition of intracellular mass dense concretions.

Table 4.13 displays the mean concentrations of the elements present in the mass dense concretions of tubule cells stimulated with 1 mM dibutyryl cAMP. The pattern of elemental distribution in type A concretions altered considerably in response to incubation in DB-cAMP containing saline. Type A concretions from DB-cAMP stimulated cells contained high concentrations of P and Ca, present in the approximate ratio of 1:1 (r^2 = 0.950, P<0.05). However, P and Ca mean concentrations were lower than control type A concretions (t=2.755, P<0.05 and t=2.687, P<0.05 respectively). The values were 1307.1 ± 219.9 mmol Kg^{-1} d.w. and 1073.5 ± 163.2 mmol Kg^{-1} d.w. respectively. The mean Cl concentration (446.3 ± 2 mmol Kg^{-1} d.w.) was higher than in control type A dark bodies (t=3.366, P<0.05), but the Na concentration (448.7 ± 61.7 2 mmol Kg^{-1} d.w.) was not significantly different (t=1.543, P>0.05). This rise in the chloride content was the one most notable difference between DB-cAMP stimulated and control type

Table 4.13. The elemental composition and mass density of mass dense concretions in tubules incubated in control saline plus 1 mM DB-cAMP. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean.

			Elemental concentration (mmol Kg^{-1} dry weight)						
Concretion	Mass density	*n*	K	Na	Cl	P	S	Mg	Ca
A type	1.83	11	62.0 ± 11.7	448.7 ± 61.7	446.3 ± 42.8	1307.1 ± 219.9	87.4 ± 12.1	191.2 ± 49.9	1073.5 ± 163
B type	1.25	10	191.2 ± 16.8	221.2 ± 65.8	177.5 ± 43.9	115.5 ± 47.5	384.9 ± 44.9	65.1 ± 21.8	*
C type	-	-	-	-	-	-	-	-	-

A concretions. The concentrations of Na and Cl displayed a statistically significant rectilinear relationship (r^2 = 0.851, P<0.001). The concentration of K (62.0 ± 11.7 mmol Kg^{-1} d.w.) was substantially less than recorded in control type A concretions (t=8.576, P<0.001), and was over 4 times lower than the mean central cytoplasm K concentration in DB-cAMP stimulated cells. The mean Mg concentration of 191.2 ± 49.9 2 mmol Kg^{-1} d.w. was similar to the control readings, as was the S concentration (87.4 ±12.1 2 mmol Kg^{-1} d.w.). Finally, the type A bodies were approximately 27% less dense than their counterparts in control saline-incubated Malpighian tubule cells.

Unlike type A concretions, DB-cAMP treated type B concretions retained a similar elemental "signature" to the control cell bodies. Like control type B mass dense concretions, S and Na were found to be the dominant elements. Both S and Na were present in similar mean concentrations (t=1.559, P>0.05 and t=0.310, P>0.05 respectively) in control and DB-cAMP stimulated concretions. The concentrations of these two elements were 384.9 ± 44.9 and 221.2 ± 65.8 2 mmol Kg^{-1} d.w. respectively. Like control type B concretions, no significant rectilinear relationship was present between the concentrations of Na and S (t=0.045, P>0.05). The mean concentrations of Cl did not differ in control and DB-cAMP stimulated cells (177.5 ± 43.9 2 mmol Kg^{-1} d.w.) (t=0.347, P>0.05). As observed in control cells, P, Mg and Ca were present at low concentrations (approximately 116, 65 and below its detection limit respectively). In general, Na, K and Cl concentrations were close to those seen in the DB-cAMP stimulated cytoplasm, but the S content of these concretions was greater. Type B concretions were only 52% of the mass density of the control type B bodies. No C-type concretions were recognised in DB-cAMP stimulated tubule cells.

The effect of corpora cardiaca extract on the composition of intracellular mass dense concretions.

Mean elemental concentrations from concretions in tubules cells stimulated with corpora cardiaca gland extract are presented in table 4.14. No concretions recognisable as type A were discerned in corpora cardiaca-treated tubule cells.

A number of concretions were noted in corpora cardiaca stimulated tubules which were characterised by high concentrations of S, Na and K,

Table 4.14. The elemental composition and mass density of mass dense concretions in tubules incubated in corpora cardiaca extract. Concentrations in mmol Kg^{-1} dry weight, values are means ± standard error of the mean.

			Elemental concentration (mmol Kg^{-1} dry weight)						
Concretion	Mass density	*n*	K	Na	Cl	P	S	Mg	Ca
A type	-	-	-	-	-	-	-	-	-
B type	1.41	7	359.7 ±48.8	222.4 ± 53.7	66.8 ± 10.3	58.4 ± 25.1	362.0 ± 60.9	112.2 ± 24.6	*
C type	1.79	24	281.2 ± 14.9	92.0 ± 13.1	34.3 ± 5.3	64.0 ±14.4	55.3 ± 3.2	227.3 ± 22.4	*

conforming to the distribution of elements in type B concretions. The mean S concentration in the C.C. extract-stimulated type B concretions (362.0 ± 60.9 mmol Kg^{-1} d.w.) was not significantly different from the control values (t=0.703, P>0.05). Additionally, the Na content of these B type concretions closely matched that seen in control cells; the mean concentration being 222.4 ± 53.7 mmol Kg^{-1} d.w. (t=0.319, P>0.05). However, the chief difference between the control and C.C. extract-stimulated type B bodies was with regard to potassium. The K content was higher than the control type B mass dense bodies (t=3.747, P<0.05), and was approximately 360 mmol Kg^{-1} d.w., similar to C.C. stimulated cells' cytoplasmic K concentrations. Cl, Mg, P and Ca were not present in these bodies in large concentrations, (approximately 67, 112, 58 mmol Kg^{-1} d.w. and below its detection limit respectively) as noted in control cell type B bodies. Mg and Cl appeared in similar concentrations as seen in the cytoplasm. The mass density of the type B bodies was considerably lower than that seen in control type B concretions, it was 1.41.

The second type of concretion detected, the type C, was distinguished by high concentrations of Mg and K. The concentration of these two elements were 277.3 ± 22.4 and 281.2 ± 14.9 mmol Kg^{-1} d.w. respectively, In the C.C. extract-stimulated cells, the mean concentrations of Mg did not differ from the appropriate control value (t=0.476, P>0.05), though the K concentration was significantly greater (t=2.323, P<0.05). Like control type C mass dense inclusions, the Mg and K concentrations did not display a significant rectilinear relationship (r^2 = 0.001, P>0.05), though in both cases, the range of concentrations were approximately the same. A drop in the mean Na concentration (t=2.619, P<0.05) of type C concretions in this tissue is apparent if control and corpora cardiaca extract treated type C concretions are compared. The Na concentration in the latter was only 92.0 ± 13.1 mmol Kg^{-1} d.w. P, Cl, S and Ca were present in low mean concentrations, as observed in tubule cells incubated in control saline. The concentrations of these elements were approximately 64, 34, 55 mmol Kg^{-1} d.w. respectively; Ca was below its detection limit. The mass density of this concretion type was 1.79, so like the corpora cardiaca extract stimulated type B mass dense bodies, type C concretions remained structures in which elements were concentrated above the level which would be observed a similar area of cytoplasm.

4.4. Discussion.

4.4.1. Intracellular elemental composition.

Control saline.

Dry weight elemental concentrations from Malpighian tubule cells incubated in control saline indicated that the intracellular distribution of potassium was not uniform (Table 4.4). A gradient of this element was present, increasing in concentration from basal to apical surface. This gradient was apparent in the calculated wet weight concentrations for K (Table 4.5). Since Na dropped below its detection limit in the apical region of the cell, it was not possible to clearly identify a gradient in this element's distribution. The intracellular concentrations of Cl, P and Mg displayed no such gradients. S concentrations differed significantly between some of analysed regions, but no clear gradient existed. Ca concentrations were close to the detection limit of this element and relatively constant between intracellular sites.

The basement membrane was dominated by high concentrations of Na and Cl. In comparison with the bathing saline which this region of the cell was in intimate contact with, the concentrations of K and Mg were higher than might be expected; high K concentrations were reported at the basement membrane in previous studies on these cells (Pivovarova *et al.* 1994a) and in the basal lamina of other epithelial cells (Gupta & Hall 1979, 1981). It is possible that the polyanionic character of the basement membrane enables this area to act as a sink for cations such as K^+ (Gupta 1989), enabling them to be recycled back to the basolateral Na^+-K^+-ATPase. High levels of S observed in the basement membrane region were also reported in earlier analyses of *Locusta* tubule cells (Pivovarova *et al.* 1994a), which may reflect the high sulphur content of the basal lamina proteoglycans and glycoproteins, especially heparan sulphate (Gupta 1989). There was however general agreement in the dry weight elemental distribution at the basement membrane between this and earlier studies. Both reported high concentrations of Na and Cl at this site, with Cl being slightly lower than Na (Pivovarova *et al.* 1994a). K and P were present in low concentrations in the basement membrane, with a higher S concentration (Pivovarova *et al.* 1994a). Ca was above the detection limit at the basement membrane in this and earlier studies (Pivovarova *et al.* 1994a). Pivovarova *et al.* recorded a

concentration of Mg which was below its detection limit at this site, in contrast to this investigation (Pivovarova *et al.* 1994a). In terms of absolute concentrations, this investigation noted a basement membrane mean Na concentration approximately 100 mmol Kg^{-1} dry weight greater than that of Pivovarova *et al.* (1994a), and a mean Cl concentration approximately 150 mmol Kg^{-1} dry weight greater.

Relatively large standard deviations were associated with the intracellular concentrations recorded in this study. This is a noted feature of dry weight X-ray microanalysis data in Malpighian tubules and other secretory epithelia (Pivovarova *et al.* 1994a,b; Wessing & Zierold 1996; Gupta *et al.* 1978; Gupta *et al.* 1980). This is considered to primarily reflect inhomogeneity in intracellular elemental distribution on the small spatial scales over which microanalysis occurs, rather than errors in quantitation (Pivovarova *et al.* 1994a). Additionally, it was found in this study that the nature of the cryosectioned tubule cells made it difficult to exclude extracellular fluid from analysis of the basal infoldings and the apical microvilli. This was because of the relatively small cross-sectional area of an individual infolding or microvillus, in comparison with the diameter of the probe beam. Analysis of these zones of the cells required positioning the beam either immediately below the basement membrane or immediately above the luminal space (recognisable by the presence of protozoan parasites *Malameoba locusta*), thus inevitably including the adjacent extracellular contents, since tissue shrinkage during preparation made it impossible to separate these areas from the infoldings or microvilli. This difficulty has been alluded to in earlier microanalytical studies in this epithelia (Pivovarova *et al.* 1994a), though cryoprotectants such as dextran have been used elsewhere to retain better ultrastructural preservation of extracellular spaces (e.g. Gupta *et al.* 1976; reviewed in Warley 1997).

Overall, mean dry weight intracellular concentrations of K, Na and Cl differed from earlier values given for this tissue (Pivovarova *et al.* 1994a,b). The K concentrations values in Table 4.4 were about 120 mmol Kg^{-1} dry weight less than the previously noted concentration at the equivalent site (Pivovarova *et al.* 1994a,b) and the Na concentration in the basal and central cytoplasmic regions were greater. The Cl concentrations, particularly at the basal and apical edges of the cell were also higher. Both the P and S concentrations are comparable between the investigations. These difference are reflected in the estimated concentrations of K, Na and Cl in mmol l^{-1} wet weight (Table 4.5). The mean wet

weight concentration of K was 30 to 40 mM lower than that calculated by Pivovarova *et al.* (1994a) in these cells; for example, the apical microvilli concentration was estimated as approximately 160mM in this study, compared with the earlier estimate of approximately 214mM (Pivovarova *et al.* 1994a). The calculated central cytoplasm Cl concentration was about 20mM greater in this study, but it was not possible to quantify the magnitude of difference between the two investigations' Na concentration since this was below the detection limit in the earlier work (Pivovarova *et al.* 1994a). The intracellular concentration of Cl was considerably lower than the sum of Na and K concentrations, probably because the majority of intracellular macromolecules and metabolites are negatively charged, hence these fixed charges make good this deficit, maintaining a Donnan equilibrium (Alberts *et al.* 1989).

These findings are in agreement with previous work by Pivovarova *et al.* (1994a,b) in *Locusta* tubule cells. In these two studies, concentration gradients of K were also reported, increasing from the basal to apical surface (Pivovarova *et al.* 1994a,b). Phosphorus was evenly distributed through the cytoplasm, and low Ca concentrations were also noted in the cytoplasm (Pivovarova *et al.* 1994a,b). Significantly, the distribution of Cl was also similar, with no clear gradient present, though a statistically significantly lower central cytoplasm Cl concentration was observed in comparison with the apical cytoplasm and apical microvilli (Pivovarova *et al.* 1994a). In the present study, only the apical microvilli concentration was significantly greater than the central cytoplasm Cl concentration.

The major difference between the data presented here and earlier findings concerned sodium. In this study, Na was detected intracellularly, in contrast to the findings of Pivovarova *et al.* (1994a,b), who reported that the Na concentration was below the detection limit (approximately 50 mmol Kg^{-1} d.w, or 17mM) at all measured sites intracellularly. Pivovarova *et al.* (1994a) maintained the tubules under different temperature and humidity conditions during dissection and immediately prior to the experimental incubation period, which may account for the difference in Na content. It is pertinent to note that the temperature sensitivity of some transport enzymes and fluid secretion by Malpighian tubule cells has been previously emphasised (Anstee & Bowler 1979).

The control mM concentrations of K and Cl are however in agreement with other estimates in this tissue. Intracellular microelectrode measurements

(Morgan & Mordue 1983b) suggested an intracellular K concentration of 95mM, close to the central cytoplasm estimate of 116mM in this study, and an intracellular Cl concentration of 51mM, again close to the values presented in Table 4.5. These workers reported measurements of the concentrations of Na^+, K^+ and Cl^- using ion selective microelectrodes; they noted a K^+ concentration that was broadly similar to that found in this study using AAS (see chapter 3), but an Na^+ concentration was lower (Morgan & Mordue 1983b). On the other hand, estimates of the intracellular [K^+] by Nerstian plots gave a concentration of approximately 165mM in *Locusta* Malpighian tubule cells (Baldrick *et al.* 1988).

Intracellular elemental content has been studied in other insect secretory epithelia. Unstimulated larval *Drosophila hydei* Malpighian tubules have been probed using X-ray microanalysis, and gradients of K and Cl discerned when expressed either as dry or wet weight values (Wessing & Zierold 1993, 1996). The intracellular Cl concentration was similar to that measured in *Locusta* tubule cells in this study, for example the apical microvilli concentration was approximately 180 mmol Kg^{-1} d.w. or 46 mmol Kg^{-1} water (Wessing & Zierold 1993, 1996). Moreover, the dry weight content of K in the intermediate cytoplasm of 337 mmol Kg^{-1} d.w. was close to the central cytoplasm value noted in Table 4.4., with a wet weight value as measured by dark field intensity of 156mmol Kg^{-1} w.w. The gradient of K was substantial with an apical microvilli concentration of some 350 mmol Kg^{-1} water (Wessing & Zierold 1993, 1996). Differences in the concentration of elements in the posterior and anterior tubules in *Drosophila* larvae have been reported; the former's [K] was 154mmols Kg^{-1} wet weight (which equates to approximately 205mM) and the latter's 103 (137mM), whilst the [Cl] were 19 (25mM) and 14mmols Kg^{-1} wet weight (19mM) respectively (Zierold & Wessing 1990).

An earlier investigation into elemental distribution in *Rhodnius* Malpighian tubules, also using X-ray microanalysis, indicated that unstimulated tubules also displayed gradients of K and Cl (Gupta *et al.* 1976). Cryosections were analysed in both an hydrated and frozen-dried state to calculate directly the elemental concentrations in mmol l^{-1}. Overall, a gradient which matched that seen in *Locusta* tubule cells was seen in the wet weight concentration of K, increasing from the basal to apical surface with a central cytoplasm concentration close to that reported here, of 103±2mM (Gupta *et al.* 1976). A similar pattern in Cl distribution was also noted, displaying a low value in the central cytoplasm, but

higher concentrations at the basal and apical regions of the tubule cells (Gupta *et al.* 1976). The central cytoplasmic Cl concentrations were remarkably similar, estimated at being 28.8±2.8mM in *Locusta* tubule cells (see Table 4.5) and 31±4mM in *Rhodnius* (Gupta *et al.* 1976). Na concentrations were lowest in the central cytoplasm region in *Rhodnius* tubules, though like Cl were higher in the apical and basal areas (Gupta *et al.* 1976). Measurements of the elemental concentrations along the cytoplasm of the long apical microvilli indicated decreasing concentrations of Na and Cl, and an increasing K concentration, which at the tip of the microvilli was close to the value estimated at the apical microvilli in this study, approximately 160mM (Gupta *et al.* 1976). A similar anion deficit was present in both *Rhodnius* (Gupta *et al.* 1976) and *Locusta* apical microvillar region, with a markedly lower Cl concentration present than K. X-ray microanalysis readings of Na and K elemental concentrations from unstimulated *Rhodnius* tubule lumen were in close agreement with the fluid ion concentrations of Na^+ and K^+ collected by AAS in this and earlier studies on *Locusta*, as well as ISM readings (Morgan & Mordue 1983b; Pivovarova *et al.* 1994a; Al-fifi *et al.* 1998a). However, ISM recordings on *Locusta* tubule fluid indicated a higher Cl^- concentration (Morgan & Mordue 1983).

In the absorptive midgut epithelia of the anterior caeca in the desert locust, *Schistocerca gregaria* (Dow *et al.* 1981), intracellular [Na] was approximately 11mM, or 42 mmol Kg^{-1} dry weight, whilst [K] was approximately 133mM (625 mmol Kg^{-1} dry weight) (Dow *et al.* 1981). [Cl] was 25mM (240 mmol Kg^{-1} dry weight), and [P] was 960 mmol Kg^{-1} dry weight (Dow *et al.* 1981). Ca and Mg concentrations were low, but a considerable concentration of S was present intracellularly, approximately 245 mmol Kg^{-1} dry weight (Dow *et al.* 1981). These data indicate this tissue follows broadly similar trends to those observed in this study of *Locusta* Malpighian tubules. Dow *et al.* reported that a consensus emerged from the literature regarding intracellular ion concentrations, with EDS and AAS data suggesting that both Na and Cl were less than 40mM, whilst K was around 150mM, and the estimated dry weight of the cells was between 15 and 25% (Dow *et al.* 1981). Later, Dow *et al.* (1984) confirmed that goblet and columnar cells from *Manduca sexta* posterior midgut possessed K concentrations in the range 96-146mM, whilst Cl concentrations were 20-30mM. The intracellular concentration of sodium was essentially zero in this tissue (Dow *et al.* 1984). *Calliphora erythrocephala* salivary glands were estimated from X-ray

microanalysis data to have a [K] of 135mM, confirmed by ion-selective electrode measurements as 143±4mM; [Cl] was approximately 33mM, whilst [Na] was 20mM (Gupta *et al.* 1978). Additional measurements using ISM indicated an intracellular [K] in the range 105-140mM in this tissue (Berridge and Schlue 1978), with an earlier estimate being slightly higher at 190mM (Berridge *et al.* 1976). In the same species, rectal papillae were shown to display [Na] of 23mM, [K] of 85mM and [Cl] of 28mM (Gupta *et al.* 1980). Leyssens *et al.* measured the $[K^+]_i$ in the Malpighian tubules of the *Formica polyctena* using ion-selective microelectrodes and obtained a value of 82mM, with a range of between 58 and 128mM recorded, though it was noted that this value may have been an overestimate (Leyssens *et al.* 1993a). Finally, a later investigation in the same tissue and species, also using ISM, gave a $[Cl]_i$ of 50mM (Dijkstra *et al.* 1995). The range of concentrations of K^+ and Cl^- reported in the literature were reviewed in these last-mentioned studies on *Formica*, and the general agreement between such studies noted (Leyssens *et al.* 1993a; Dijkstra *et al.* 1995).

This brief review of the intracellular elemental concentrations measured in insect epithelial cells suggests that the findings in this study do not substantially differ from those described elsewhere. The magnitude of the elemental concentrations reported here, and the gradients in the concentrations observed of some elements are common to other studies. Wherever possible, data from earlier investigations have been given in units of mM to permit easy comparison between studies. However, as is well recognised, X-ray microanalysis usually provides data in the form of concentrations per unit dry mass (i.e. mmol Kg^{-1} dry weight). Measurements of intracellular elemental concentrations in these units are of restricted use *per se* in physiological terms (Gupta 1993). In Table 4.5, the conversion of microanalysis concentrations of Na, K and Cl from mmol Kg^{-1} dry weight to mmol l^{-1} (i.e. mM) (Pivovarova *et al.* 1994a) is based on two assumptions. Firstly, that the cytoplasmic cellular dry mass of the cells is approximately 25%; this estimate was proposed by Dow *et al.* (1981) in *Schistocerca* midgut caeca, and a survey by Gupta of a wide range of both vertebrate and invertebrate cells indeed suggests that the range of dry mass fractions of the cells wet mass is between 20 and 25% (Gupta 1993). Secondly, it is supposed that Na, K and Cl all exist within the cell in a free, ionic form (Dow *et al.* 1981; Gupta *et al.* 1976); microanalysis only distinguishes between nuclides and gives no information on the bound or unbound state of the element analysed

(Warley 1997). By comparing the ISM-derived intracellular concentrations of K and Cl in *Locusta* tubule cells (Morgan & Mordue 1983b) and those obtained by X-ray microanalysis in this study, this assumption also appears reasonable for these elements; however, in *Calliphora* salivary gland, a comparison between ISM and X-ray microanalysis data suggested that a large fraction of the [Cl] may have been bound (Gupta *et al.* 1978), though the close agreement between the two techniques' [K] values did suggest this element was present chiefly in the form of its cation.

On the basis of the general similarity between the ISM values of Morgan and Mordue (1983b) and those presented in Table 4.5, the estimates in this table seem to represent accurately the elemental concentrations of Na, K and Cl in mM. P, S. Mg and Ca are unlikely to be present as free ions, and it has been suggested that Mg and Ca may be bound to organic molecules composed of P and S in *Locusta* tubule cells (Pivovarova *et al.* 1994a). The internal osmolarity of the Malpighian tubule cell has been estimated from the sum of the dry weight concentration of Na and K, and it is thought that this is a reliable indicator of the cell-water content of the cell (Pivovarova *et al.* 1994a). A decrease in the sum of Na, K and Cl, and a fall in the concentration of P, plus a drop in the dry weight fraction have been taken as good indicators of cell swelling in toad urinary bladder (Rick & Schratt 1989).

In summary, incubation of *Locusta* Malpighian tubules in control saline results in elemental distributions and concentrations, particularly of K, Na and Cl, which are consistent with previous findings, recorded using not only X-ray microanalysis but AAS and ISM, in a range of insect epithelia cells. In conjunction with ISM data (Morgan & Mordue 1983b), these data suggest that K and Cl exist in a free ionic form in control incubated tubule cells. It is unclear exactly how the apparent gradient of K arises in cells such as Malpighian tubules which undergo rapid fluid and ion turnover (Maddrell & O'Donnell 1992), but an intimate connection between elemental gradients and transcellular water flow has been established in toad urinary bladder (Rick & Schratt 1989). The contribution of this "solvent drag" to the observed ion distribution in *Drosophila* tubule cells has been questioned, and it has been suggested that intracellular cell constituents such as glycosaminoglycans and proteoglycans may be responsible for the distribution of cations and anions (Wessing and Zierold 1992). It is suggested that measured intracellular levels of P, S and Ca in this study may be due to their presence as

constituents of proteins, nucleic acid such as RNA, metabolites, for example ATP, or even second messenger molecules such as IP_3 or cAMP, found in the cytoplasm of Malpighian tubule cells.

Control saline plus 0.1% DMSO.

This experimental treatment was used as a control to assess the intracellular effect of the furosemide solvent DMSO on elemental distribution. In *Locusta* Malpighian tubule cells incubated in 0.1% DMSO, only chlorine exhibited a systematic difference from the control mmol Kg^{-1} dry weight values (see Table 4.7). Equally, the basement membrane elemental concentrations were closely matched. Aside from a number of isolated readings chiefly in the basal region of the cells, the main difference was noted in the Cl concentrations. Though considerably lower than control cell readings, the [Cl] showed no overall gradient in distribution. The decrease in Cl may be an artefact of microanalysis, since Cl (and also S) may be selectively lost from biological tissue at relatively low radiation doses (above 10^7 e nm^{-2}), in spite of cooling the specimen to liquid nitrogen temperatures (Cantino *et al.* 1986). It is possible that such selective loss of Cl occurred in this cryosectioned material. The fact that only the Cl level has been affected may support this suggestion. The particular sensitivity of halogens to mass loss during irradiation of organic material is well known (Chandler 1977). Further, the retention of a Cl concentration similar to control values at the basal infoldings is suggestive of a non-systematic change in intracellular Cl content. Aside from this, DMSO had no substantial effect upon elemental distribution and concentrations in *Locusta migratoria* Malpighian tubules. Since DMSO did not alter the rate of tubule fluid production or the cationic composition of this fluid (see chapter 3), it seems reasonable to conclude that this compound had no significant effect on the functioning or content of the tubule cells.

Inhibitors of fluid production.

Furosemide.

Incubation of *Locusta* Malpighian tubule cells in saline containing 1 mM furosemide resulted in substantial changes in the distribution of K, Na, Cl and P intracellularly (see Table 4.8). In contrast, S and Mg concentrations were similar to control cell values in the majority of the sites measured, and intracellular Ca

was low throughout the cell. Essentially, furosemide did not alter the pattern of distribution of Mg and S in the cells. The basement membrane K and Cl concentrations were significantly greater than the control cell values for this site; Na was not. The K concentration was lowered throughout the cell and the clear gradient noted in control cells was removed. The K concentration was lowered by between 50-220 mmol Kg^{-1} d.w., depending on the site considered. There was an increase in the concentration of Na at all the intracellular sites measured; this increase was maximal at the central cytoplasm, and was approximately 260 mmol Kg^{-1} d.w. Chloride concentrations were higher at all but two cell sites in comparison with control tissue. The apical microvillar border Cl concentration was in fact lower than the control at this site by approximately 90 mmol Kg^{-1} d.w, and the data displayed a partial gradient, decreasing in concentration from the central cytoplasm. The P concentrations were also lower in all but one area, but the cytoplasmic distribution was similar to that seen in control tissue.

Reports of the effect of loop diuretics on intracellular elemental concentrations in any tissue are quite limited. A number of investigations have recorded a fall in intracellular [K] (Saga & Sato 1989), and also a decrease in [Na] and [Cl] (Mörk *et al.* 1992), as a result of loop diuretics inhibition of vertebrate epithelia. Other studies have shown that loop diuretics had no effect on the intracellular [Cl] (Ussing 1982; Dörge *et al.* 1985).

In *Locusta* Malpighian tubules, X-ray microanalysis data supports the hypothesis that furosemide-sensitive basolateral Na^+-K^+-Cl^- cotransport plays a part in ionic transport. Intracellular [K] levels fell, and this element's gradient was removed, implicating the cotransporter in the maintenance of this characteristic feature of potassium distribution in the tubule cell. Further, the rise in basement membrane [K] and [Cl] support the proposed inhibition of the movement of these elements across the basolateral surface of the cell. This decrease in intracellular [K] may be because continued K^+ efflux across the apical membrane is no longer balanced by adequate K^+ influx across the basolateral membrane, as a result of furosemide inhibition of the Na^+-K^+-$2Cl^-$ cotransporter. The recorded decrease in intracellular [P] may be an indication of slight cell swelling as a result of fluid secretion inhibition by furosemide, but since the total cation concentration remained close to the control value in furosemide treated cells, the cell-water content seems likely to have been relatively unaffected in this case. Where these data differed substantially from other studies was that the intracellular

concentrations of Na and Cl in *Locusta* Malpighian tubule cells increased in response to 1 mM furosemide. The reasons for such a pattern of distribution are not immediately clear. It may be that intracellular Na^+ retention occurs in order to maintain a Donnan equilibrium within the cell. A rise in the level of Cl may reflect its action as a counterion for the increased intracellular Na concentration. Like control tissue, the furosemide-inhibited tubule cells [Cl] was less than that of the combined [K] and [Na], suggesting an anion deficit which is likely to be compensated for by fixed anion charges on organic macromolecules within the cell. The anion deficit (i.e. the sum of the K and Na concentrations minus that of the Cl concentration) between the control and furosemide-treated cells was of comparable magnitude at equivalent cell sites, Table 4.15 shows these data;

Table 4.15. Anion deficit in control and furosemide-treated *Locusta* Malpighian tubule cells measured using X-ray microanalysis.

Cell site.	**Anion deficit in control saline treated cells (mmol Kg^{-1} dry weight)**	**Anion deficit in furosemide saline treated cells (mmol Kg^{-1} dry weight).**
Basal infoldings	334.7	259.8
Basal cytoplasm	384.3	401.8
Central cytoplasm	433.7	331.1
Apical cytoplasm	350.0	362.9
Apical microvillar border	314.2	401.8

This suggests that the increase in Cl concentration in furosemide-inhibited Malpighian tubule cells may indeed be linked to the rise in intracellular Na, retaining a constant level of anion deficit, so that Cl^- acts as a counterion for Na^+ intracellularly. This anion has been suggested as a counterion for K^+ in *Drosophila* Malpighian tubule cells (Wessing *et al.* 1993).

In summary, furosemide caused considerable intracellular elemental change, and X-ray microanalysis data suggest Na^+-K^+-Cl^- cotransport contributes to K transport in *Locusta* Malpighian tubule cells. Changes apparent in the intracellular [Na] and [Cl] have not been seen in response to this compound previously. As the "point of entry" for intracellular ions, it is unsurprising that inhibition of transport events at the basolateral tubule cell surface can alter the

intracellular distribution of elements so dramatically, and may reflect an asymmetry in ion transport between the basal and apical surfaces under these conditions.

Bafilomycin A_1.

Intracellular microanalysis data indicated that bafilomycin A_1 also altered the nature of elemental distribution in *Locusta* Malpighian tubules. This inhibitor abolished that increasing gradient of K from basal to apical surface, although the K concentration in the basal region was considerably higher than in the control cells (see Table 4.8). A slight decrease in [K] was even seen in the apical microvilli region, though basally, the concentration was 150-180 mmol Kg^{-1} d.w. greater. An increase in the concentration of Cl in the tubule cells of similar magnitude occurred at all sites except the apical microvilli, though again, no clear gradient was observed. The intracellular [Na] decreased in the basal and central cytoplasm, and this element was below its detection limit intracellularly at nearly all sites. Intracellular levels of Mg were essentially unchanged from control values. Ca was mostly below its detection limit intracellularly, but the recorded S concentrations were higher in most regions, though these values displayed no pattern in their distribution. The chiefly unchanged P content of the bafilomycin-treated tubule cells, and the similar levels of total cations present intracellularly in comparison with control cells suggests limited changes to the cell water content in response to this inhibitor. This may not be the case at the apical microvilli, where the decrease in both [P] and [K] may reflect some swelling of the brush border region. In contrast, the somewhat higher [P] in the basal cytoplasm could indicate that some shrinkage is occurring in this area. An increase in the basement membrane K concentration, in conjunction with the elevated concentrations of K in the basal region of the cell, suggests a possible increase in passive K^+ exit down its concentration gradient.

Bafilomycin A_1 has been utilised at a concentration of 5μM to inhibit fluid production by larval *Drosophila hydei* anterior proximal Malpighian tubules (Wessing *et al.* 1993). In that study, the inhibitor was injected into the larvae *in vivo* with subsequent dissection and cryofixation occurring after a 10 minutes period (Wessing *et al.* 1993). It was reported that bafilomycin caused a decrease in the dry weight concentration of K in all cell sites, but the substantial loss of water from the cell and visible decrease in the cell volume caused a rise in the K

concentration in wet weight terms in all tubule cell sites, except the apical microvilli (Wessing *et al.* 1993). In comparison with the data recorded in this study, notable differences occur; the pattern of K dry weight distribution is reversed, with the maximal concentration at the apical surface and the lowest at the basal surface. Here, the highest concentration was observed in the basolateral region. [K] in the control-saline injected *Drosophila* tubule cells were somewhat higher in dry weight terms than in this study, but wet weight values bore comparison with estimated [K] concentrations in mM in *Locusta* tubules cells noted here; for example the intermediate cytoplasm [K] was 440±18 mmol Kg^{-1} d.w. (116±16 mmol Kg^{-1} w.w.). The difference between the wet and dry weight values was attributed to the presumed loss of K^+ via basal channels, plus the effect of the cell water loss and cell shrinkage which occurred on inhibition with bafilomycin (Wessing *et al.* 1993). Amiloride was also used in that investigation, to inhibit the proposed apical nH^+/K^+ antiporter; no loss of intracellular water was noted in this case, and the K content was significantly less in wet weight terms in the basal area of these cells, but was larger at the apical microvilli (Wessing *et al.* 1993). In the central cytoplasmic zone, the K content was unchanged (Wessing *et al.* 1993). Subsequent inhibition with the K^+-channel blocker Ba^{2+} suggested that this decrease may have been due to basolateral efflux of K^+ under inhibition with amiloride, since a combination of the two inhibitors caused a rise in the wet weight concentrations of K throughout the tubule cell site analysed with X-ray microanalysis (Wessing *et al.* 1993). ISM measurements also indicate a rise in $[K^+]$ when these tubule cells are treated with $BaCl_2$ (Wessing *et al.* 1986).

There are relatively few additional reports on the effect of bafilomycin on insect epithelia cells, but the V-type ATPase inhibitor NEM has been used in a number of studies. NEM inhibits the proton pumping V-type ATPase, as does bafilomycin A_1, but is thought to inhibit P-type ATPases at lower concentrations than have been observed to be inhibitory with bafilomycin A_1 (Bowman *et al.* 1988; Ehrenfeld & Klein 1997; Al-fifi *et al.* 1998a). In *Locusta*, 1µM NEM increased the [K] concentration intracellularly at most sites as measured by X-ray microanalysis (Pivovarova *et al.* 1994b) but did not change either the Na, Cl or P concentrations. The increase in [K] resulted in an accentuated gradient, increasing in concentration from basal to apical surface (Pivovarova *et al.* 1994b). In *Aedes aegypti*, the oxidative phosphorylation uncoupler dinitrophenol

(DNP)(10^{-4}M) caused rapid and reversible depolarisation of V_b, V_a and the TEP (Pannerbecker *et al.* 1992), so that the TEP was close to 0mV. DNP abolished the apical proton gradient by acting as a proton ionophore (Alberts *et al.* 1989). A very similar effect on Malpighian tubule cell membrane potentials was noted in *Drosophila* tubule cells on inhibition with 10μM bafilomycin A_1 (Wessing *et al.* 1993). Ion selective microelectrode measurements demonstrated that 2×10^{-4}M DNP caused a significant fall in the intracellular K concentration, from 141±8 mM to 93±6mM in *Formica* (Leyssens *et al.* 1993b). In *Formica*, both bafilomycin A_1 and NEM inhibit fluid production and depolarise V_a (Weltens *et al* 1992; Dijkstra *et al.* 1994). In *Locusta* 1μM bafilomycin and 15μM NEM also depolarised V_b (Marshall 1995). Bafilomycin A_1 decreased the intracellular pH in *Drosophila* Malpighian tubules by 0.3pH units, a result which was reversibly mimicked by the ATP synthesis inhibitor KCN (Wessing *et al.* 1993), Amiloride was seen to increase the luminal pH, as the V-type ATPase-generated proton gradient was not dissipated in exchange for K^+ via the action of the apical nH^+/K^+ antiporter (Wessing *et al.* 1993). Clearly, these results support the presence of an apical V-type ATPase in Malpighian tubule cells, which acts as a major power source for both apical and basolateral membrane potentials (Pannabecker *et al.* 1993).

As noted in control and furosemide treated cells, an anion deficit is again present in the bafilomycin-incubated cells. Table 4.16 shows that the size of this is reduced.

Table 4.16. Anion deficit in control and bafilomycin A_1 treated *Locusta* Malpighian tubule cells measured using X-ray microanalysis.

Cell site.	**Anion deficit in control saline treated cells (mmol Kg^{-1} dry weight)**	**Anion deficit in bafilomycin A_1 treated cells (mmol Kg^{-1} dry weight).**
Basal infoldings	334.7	294.0
Basal cytoplasm	384.3	148.5
Central cytoplasm	433.7	174.5
Apical cytoplasm	350.0	245.2
Apical microvillar border	314.2	149.9

The rise seen in the intracellular [Cl] is in contrast to the fall in [Na] and is not entirely mirrored by an increase in [K] at all cytoplasmic sites; this may reflect a reduction in passive anion efflux on inhibition with bafilomycin whilst basolateral influx is unchanged.

To conclude, bafilomycin A_1 causes a rise in intracellular [K] measured by X-ray microanalysis at the basal cell sites, but a decrease in the apical microvilli zone. This finding again highlights a probable uncoupling of ion movement across the apical and basolateral membranes. It is likely that inhibition of the apical V-type ATPase limits K^+ transport into the tubule lumen, in spite of its continued entry into the tubule cell across the basal membrane, leading to a rise in the K concentration intracellularly. A fall in the intracellular [Na] is noted in most cell sites, perhaps reflecting an increase in the exit of Na^+ basally, so again maintaining intracellular osmotic balance. What is clear is that bafilomycin A_1 initiates changes in the distribution of elements which firmly implicate V-type ATPase activity in K^+ transport, and suggest that events at the apical surface can indirectly modify ion movement basally, for example, an increase in passive K^+ efflux basolaterally down its concentration gradient.

Rb-saline.

X-ray microanalysis readings taken from cells incubated in K^+-free, Rb^+-containing saline displayed a dramatic reduction in intracellular K concentrations (see Table 4.9). In spite of this decrease, and in spite of a limited number of sites available for analysis, the increasing gradient in K concentration observed in control-saline incubated cells was retained. Rubidium was identified within the cells, and a gradient in this elements distribution was present in the cell. The concentration of rubidium was however less than that of K in control saline-incubated tubule cells. The Na concentrations were unchanged at the basal infoldings, but lower at the central cytoplasm site, than the control values. Conversely, the apical microvillar Na concentration was higher in the Rb incubated tubule cells. P, S and Mg concentrations were mostly unchanged from the appropriate control values, and Ca was below its detection limit throughout the cell. Cl was present at a lower concentration than seen in control cells in all sites, though a similar pattern was observed in its distribution, with a low central cytoplasm concentration in comparison with the apical microvillar value. The drop in Cl concentration was between approximately 50 and 100 mmol Kg^{-1} d.w. The

similarity between the Rb and Cl pattern of distribution strongly suggests that Cl is acting as a counterion to Rb in the Rb cells. The general decrease in chloride in the Malpighian tubule cells incubated in rubidium treated may not be artefactual, as has been suggested for the fall in intracellular [Cl] detected in the control incubation with 0.1% DMSO. The low level of Cl in all the available sites for analysis in Rb inhibited cells contrasted with the DMSO control, since the latter exhibited a basal infolding concentration not significantly different from the control value.

A previous study using Rb^+ in place of K^+ in *in vitro* bathing saline also noted similar effects on intracellular elemental distribution in *Locusta* Malpighian tubule cells (Pivovarova *et al.* 1993, 1994a). A gradient in the concentration of K was seen intracellularly, though the concentration was reduced in magnitude at all sites. The actual concentration of K present is very close in both studies (Pivovarova *et al.* 1994a). A gradient in the distribution of Rb was reported which displayed a very similar pattern to that found in this study (Pivovarova *et al.* 1994a). The intracellular rubidium concentration in this study is slightly lower than noted previously, but the total cation content (Rb plus K plus Na) is also lower than that in control-saline incubated tubule cells (K plus Na). This was suggested to indicate a fall in the cell-water content under these conditions (Pivovarova *et al.* 1994a). Even though in this investigation the Cl concentration in Rb-incubated tubule cells is much lower than control tissue, the anion deficit is reduced in comparison, since the total concentration of cations at any of the measured sites is also greatly reduced. Though both these investigations show that incubation in Rb-saline did not affect intracellular P concentrations, two main sources of difference in these studies are indisputable. An overall rise in intracellular Na concentration throughout the cell sites measured was reported earlier, and no change was observed in intracellular Cl levels (Pivovarova *et al.* 1994a). Cl levels fell in this study, and an increase in Na was only recorded in the apical microvilli. When the period of incubation in Rb-containing saline was increased to 45 minutes, the levels of intracellular Rb increased, and the gradient was retained. (Pivovarova *et* al. 1994a). The chloride concentration was seen to increase significantly in comparison with control values in the apical cytoplasm and apical microvilli as a consequence of this longer incubation period (Pivovarova *et* al. 1994a).

A number of additional studies have combined the use of rubidium and X-ray microanalysis to probe ionic transport. Frog skin epithelium principal cells were demonstrated to take up Rb when incubated in Rb-containing saline (i.e. K^+-free), and though the cells retained a considerable K content, the concentration of this element was reduced from approximately 115 to 63.3 ± 3.8 mmol Kg^{-1} wet weight (Dörge *et al.* 1989). Incubation with saline containing the inhibitors amiloride and ouabain inhibited this Rb uptake, though amiloride alone was somewhat less inhibitory (Dörge *et al.* 1989). Rb could not fully substitute for intracellular loss of K in this cell type too, since in Rb-saline, the concentration of Rb was less than that of K in control saline (Dörge & Rick 1990). It was proposed that under normal conditions, the Rb uptake into the cell was effected through the action of the Na^+-K^+-ATPase, and that Rb passage through either the electroneutral cotransporter or K^+-channels was negligible (Dörge & Rick 1990). It was also suggested that the cotransporter operated in a Cl/Cl self exchange manner in order to explain the greater uptake of Rb in comparison with Cl, in cells incubated in an RbCl-containing saline after a period in Cl-free media (Dörge & Rick 1990). Significantly, uptake of Rb and Cl occurred when principal cells were first depleted of Cl by incubation in Cl-free saline, in the presence of amiloride and ouabain (Dörge *et al.* 1989).

Glial cell K uptake was investigated using rubidium in the leech *Macrobdella decora*, and a comparison made with uptake by neuronal cells (Saubermann *et al.* 1992). Glial cells act as buffers against increasing potassium concentrations, and eventually return K to the neuron cells (Saubermann *et al.* 1992). Differences were confirmed in the uptake rates of Rb into the two cell types; glial cells exchanged Rb for K much faster than neurons, though like *Locusta* Malpighian tubule cells, both types could replace K with Rb intracellularly (Saubermann *et al.* 1992). As observed in *Locusta* tubule cells, glial Rb distribution varied as a function of time, with an increasing concentration present intracellularly as the incubation period in Rb-containing saline was extended (Saubermann *et al.* 1992). Glial cells displayed a constant water content of about 87% when incubated in saline containing 20mM [Rb]. It was however considered unlikely that an active metabolic process was responsible for the majority of cation uptake into glial cells (Saubermann *et al.* 1992).

The data presented in this study is similar in several aspects to that reported elsewhere. Rb is evidently able to enter Malpighian tubule epithelial

cells, can substitute to some extent for the loss of intracellular K, and assumes a gradient which is similar in pattern to that of K in normal cells. The shortfall in K concentration under these conditions is not fully compensated for by intracellular levels of Rb, probably a consequence of the basolateral membrane of the tubule cells' reduced ability to allow influx of this cation in comparison with K^+. A fall in intracellular Cl content is observed, but a rise apically in Na is apparent. The fall in Cl levels may indicate "inhibition" of Na^+-K^+-$2Cl^-$ cotransport at the basolateral surface by Rb, and the rise in Na concentration could again be a consequence of the need to preserve an intracellular Donnan equilibrium.

Stimulators of fluid production.

1 mM dibutyryl cAMP.

Incubation of Malpighian tubule cells in saline containing DB-cAMP also significantly altered the distribution of Na, K and Cl (see Table 4.10). As with Rb-saline, DB-cAMP lowered the concentration of K at all the cell sites, although a gradient of this element's distribution was retained. The differences between the concentration of K in the DB-cAMP stimulated tubule cells and control Malpighian tubule cells varied from approximately 40 to 150 mmol Kg^{-1} dry weight, the largest difference occurring at the apical region of the cell. Neither furosemide nor bafilomycin A_1 treatment retained the basal to apical concentration gradient of K in *Locusta* tubule cells. Na levels were higher throughout the cell compared with control values, except in the central cytoplasm, but the Cl concentration was uniformly increased in response to DB-cAMP. No clear gradient of either Na or Cl were present, though it was noted that chlorine elemental concentrations were particularly high in the apical zone of the cell. None of the other elements displayed distributions significantly different to those seen in control cells. Unchanged P concentrations suggest little change in the cell volume occurs on stimulation with cAMP. No change in the Na, K and Cl concentrations at the basement membrane occurred in response to stimulation with cAMP.

Data collected on intracellular analysis of elemental distribution using cAMP as an exogenous stimulator of fluid production is mostly restricted to vertebrate epithelia. 8-bromo-cAMP initiated a marked decrease in the intracellular concentrations of Na, K and Cl in cultured porcine tracheal submucosal gland acinar cells, quantified by X-ray microanalysis (Zhang &

Roomans 1997). This Cl efflux could be blocked with the Cl^- channel blocker NPPB. (Zhang & Roomans 1997). It is evident that in tracheal epithelium, cAMP is sufficient to activate an apical Cl^- channel (Liedtke 1989). The human sweat gland cell line NCL-SG3 was also treated with cAMP, and a decrease in the intracellular concentration of both Cl and K observed, but this treatment had no effect on either Na or Ca concentrations (Mörk *et al.* 1996). Again, chloride channel blockers such as NPBB an anthracene-9-carboxylic acid (9-AC) prevented cAMP-stimulated Cl^- efflux (Mörk *et al.* 1996). Chloride permeability in NCL-SG3 cells may also be via Ca^{2+}-dependant channels (Whiteford *et al.* 1993). The ability of cAMP to activate basolateral Na^+-K^+-Cl^- cotransport in secretory epithelia cells has been previously noted (Xu *et al.* 1994; Regan 1995), particularly in the Malpighian tubules of *Aedes aegypti* (Hegarty *et al.* 1991). Under stimulation with 0.1mM DB-cAMP, *Aedes* tubules increase their secretion of Na^+ and Cl^-, but the rate of secretion of K^+ decreased (Hegarty *et al.* 1991).

The effect of the biogenic amine 5-HT on the intracellular elemental distribution was studied in *Rhodnius* Malpighian tubules (Gupta *et al.* 1976). In this tissue, there was a large increase in the intracellular Cl concentration in the central cytoplasm region and along the long apical microvilli, and the Na concentration was much increased at the majority of cell sites (Gupta *et al.* 1976). K concentrations were relatively unchanged (Gupta *et al.* 1976). 5-HT induces a dramatic increase in the rate of fluid secretion in the Malpighian tubules of this species, and is an *in vivo* diuretic factor (Maddrell *et al.* 1991). The effect of 5-HT therefore bears some resemblance to that of cAMP as described in this study. However, in *Locusta migratoria*, 5-HT has been reported to stimulate secretion independently of cAMP (Morgan & Mordue 1984b). An increase in the intracellular concentrations of Na and Cl accompanied the stimulation of pig acinar cells with cholecystokinin (CCK), whilst K fell, which is a similar pattern to the changes noted by stimulation of *Locusta* Malpighian tubule cells with DB-cAMP, though CCK has been implicated in a rise in Ca^{2+} levels in the cell, opening Ca^{2+}-dependant K^+ channels, as well as activating basolateral Na^+-K^+-$2Cl^-$ cotransport (Sasaki *et al.* 1996). Na^+, Cl^- and K^+ plus zymogen granules were subsequently secreted into the lumen of the glands (Sasaki *et al.* 1996).

The data presented here provides new information regarding the effect of stimulation with cAMP in insect epithelial cells. The considerable increase in Cl

concentration at the apical region of the cells suggests that Cl^- supply to the apical surface exceeds its rate of secretion into the lumen. If this is so, then it appears that cAMP differentially stimulates ion transport at the basolateral surface. Stimulated basolateral Cl^- influx into the tubule cell is very likely to be through the Na^+-K^+-$2Cl^-$ cotransporter. The increase in the intracellular Na concentration is also probably a consequence of the stimulation of this cotransporter, but the fall in intracellular K content may further highlight the incomplete nature of the stimulatory effect of cAMP. Additional stimulation of K^+ influx across the basolateral surface may be required to maintain normal $[K]_i$ under these stimulated conditions. The anion deficit is reduced, especially at the apical zone, due to the large increase in the concentration of Cl. cAMP has been clearly shown to affect Cl transport in numerous cell types, though there are difference between the effects on elemental distribution between this and previous studies using this stimulant.

Corpora cardiaca extract.

Corpora cardiaca extract did not alter the intracellular distribution of elements at the majority of the different sites analysed in Malpighian tubule cells, when compared with control incubated cells (see Table 4.11). Of all the measured elements in this treatment, Na displayed the greatest differences in its distribution, varying at most of the intracellular sites in comparison with control cells. It is suggested that this may be partly a result of an increase in the movement of Na^+ across the basolateral membrane. Mg and S levels and patterns of distribution were essentially identical to those seen in control cells. The areas that differed the most from control cells were the basal infoldings and the basal cytoplasm, but these alterations were not followed by changes in the overall characteristic patterns displayed by the concentrations of K, Cl or indeed P. The basement membrane mean elemental concentrations of Na, K and Cl were all significantly greater than those recorded from the same area in tubule cells incubated in control saline. This may derive partly from an increased level of cation cycling across this membrane, as Na^+ exits by active transport, and K^+ exits down its concentration gradient, in response to an increased rate of ion transport. The observed increase in P concentration at the basal area of the cell, plus the increase in the sum of cations, suggests that some cell shrinkage occurs in this zone of the Malpighian tubule cell. This may be linked to a general

increase in ion transport accompanying neurohormonal stimulation, and hence the solute-water coupled movement of water towards the tubule lumen.

Few other examples of elemental intracellular analysis exist with which to compare these findings. Stimulation of *Locusta* tubules with extract from the storage lobes of the corpora cardiaca glands were seen to have no significant effect upon the concentration of Na^+, K^+ and Cl^- in the tubule fluid (Morgan & Mordue 1983b), but these ISM findings did not include intracellular measurements under stimulation. Recently, corpora cardiaca extract has been reported to lower the tubule fluid [K^+] and raise [Na^+], whilst preferentially stimulating Na^+ flux into the tubule lumen (Al-fifi *et al.* 1998a). 5-HT caused similarly limited alterations in the intracellular elemental composition of *Calliphora* salivary gland, though a slight rise in the concentration of K was noted in the cytoplasm and confirmed with ISM measurements, in spite of considerable changes in the net movement of sodium and chloride across the cells (Gupta *et al.* 1978). It is interesting that stimulation of the tubule cells with an extract derived from the animal' own neurosecretory system caused so few changes in the distribution of elements, in spite of increasing the rate of fluid production and altering the composition of the fluid as mentioned above in *Locusta* (Al-fifi *et al.* 1998a). Even so, qualitative changes in ion transport events at the basal and apical surfaces cannot be excluded from occurring under such secretory conditions, especially in light of the changes noted in the secreted fluid composition.

4.4.2. Mass dense concretions.

Mass dense concretions from control saline incubated tubule cells.

The mass dense concretions observed in control saline-incubated *Locusta migratoria* Malpighian tubule cells are similar in every aspect of their elemental composition to those reported by Pivovarova *et al.* (1994a) (see Table 4.12). Three types, namely Type A, rich in P and Ca, Type B, displaying high levels of S and K, and also Na, and finally Type C, with high concentrations of Mg, K and Na were reported. The three concretion types reported in this study were in close agreement with the previous work of Pivovarova *et al.* (1994a) with respect to the concretions' mass density (in comparison with the cytoplasm), and the predominant elements in each type. In *Locusta* tubules, Pivovarova *et al*

(1994a) have suggested that the concretions may act as K stores, since under Rb-saline incubation, these structures replace K with Rb. Substantial evidence therefore suggests that the concretions seen in tubule cells are a vital part of the cells ionic economy, especially in light of the continued secretion of K^+ into the tubule lumen, in the presence of Rb-saline (i.e. K^+-free) (Pivovarova *et al.* 1994a).

As observed in other studies in this tissue (e.g. Marshall 1995), the morphology of the mass dense structure differed considerably; some appeared to be surrounded by a membrane-type structure, many were crennelated (a feature which is probably represents knife compression marks) some assumed the shape of crescents, or looked otherwise fragmented, and all, to a greater or lesser extent, were clearly darker and distinct from the surrounding cytoplasm. Some appeared to be made up of concentric layers of material (Marshall 1995). Their mass density set them apart from any other structure in the intracellular cytoplasm, either mitochondria of nucleus, and practically no section of cryofixed material lacked at least a small number of these structures.

Mass dense concretions have been observed and analysed using X-ray microanalysis in many epithelia cells, from insect and vertebrate tissue alike. In the latter, atrial specific granules are found in heart muscle tissue in rats, and are considered to be an intracellular Ca^{2+} store in atrial cardiocytes; Ca is seen at high concentrations in these structures, and Ca^{2+}-ATPase has been identified on the surface of the granules (Zhong *et al.* 1995). Calcium rich concretions are found also in the pineal glands of mammals, and in humans, these structures are known as "brain sand" or acervuli (Humbert & Pévet 1995). In rats, these concretions are characterised by high concentrations of Ca and P (Humbert & Pévet 1995). Secretory granules in mouse intestinal goblet cells were shown to contain concretions in which either S or K were present in high concentrations, depending on the cell type, with Cl, Ca, P and Na present in both cases (Ichikawa *et al.* 1994). Sulphur-rich intraluminal crystalloids were found in human prostate gland tissue (Del Rosario *et al.* 1993).

Many investigations into Malpighian tubule morphology and ontogeny have also noted the presence of dense bodies both intracellularly and intraluminally (Maddrell 1977). In *Musca domesticus*, the dense bodies accumulate electron opaque material as the individual insect ages, and in older flies, a substantial proportion of the cytoplasm of the tubule cells contained these

concretions (Sohal, quoted in Maddrell 1977). The concretions were also found in the tubule lumen, and their characteristic elemental content included P, S, Cl , Zn, Fe and Cu. It was thought therefore that their role was to act as excretory sinks particularly for these metal ions (Maddrell 1977).

Granules with a concentric, layered appearance were found in *Musca autumnalis*, and X-ray microanalysis showed that they possessed high levels of P and Ca, in similar proportions to that recorded in *Locusta* Malpighian tubule cells type A concretion (Grodowitz *et al.* 1987). In *Drosophila* larvae, concretions with very similar morphology to those reported here in *Locusta* were first reported by Wessing *et al.* (1988), who observed initially that the concretions contained Mg and Na in the ratio of approximately 1 to 5, and the concentrations of P, S and K were highly correlated with each other in these structures, though Ca was absent. The structures were enclosed in vacuoles, were connected to the ER system, and contained 3-OH-kynurenine (Wessing *et al.* 1988). The vacuoles contained glycosaminoglycans, and were concluded to be structures partially composed of inorganic and organic substances, with some inorganic cations existing in a free form (Wessing *et al.* 1988). Later work indicated however that the concretions contained Zn, not Na as reported, and that anterior larval tubules accumulated concretions with more Zn present than their posterior counterparts. The concretions were thought to have a role in the metabolism of these cells, and inhibition with ouabain caused an increase in the Zn present in these structures (Zierold & Wessing 1990). It was not known why these concretions concentrated Zn (Zierold & Wessing 1990).

Two types of concretion were identified in subsequent work. Type I were found in the distal segment of anterior tubules and contained Ca, Mg and P, whilst Type II concretions accumulated mainly P, with smaller concentrations of Mg, Ca, P and Cl. (Wessing & Zierold 1992; Wessing *et al.* 1992). This latter type originated from the microvillar region of the transitional and middle segment posterior tubules. Both Type I and II concretions were excreted into the tubule lumen unchanged during development. Investigation showed that the structure of these concretions in *Drosophila* could be manipulated by altering the diet of the insects (Wessing & Zierold 1992). A model for the intracellular role of the concretions in *Drosophila* has been recently proposed (Wessing & Zierold 1996), based on the accumulation of glycosaminoglycans in Golgi-derived vesicles in the cytoplasm, leading to the formation of the concretions. Disruption of the

Golgi-apparatus by brefeldin A led to a decrease in the intracellular K concentration, and a link between the synthesis of GAG and K transport was proposed (Wessing & Zierold 1996). The cytoplasmic vesicles were rich in GAG and were thought to accumulate positively charged ions such as K in the apical region of the cell. The vesicles were observed to eventually discharged their contents into the tubule lumen, where the GAG transformed into proteoglycans (PG), forming the so-called Type II concretions (Wessing & Zierold 1996). The GAG/cation interaction at the apical surface may form temporary ions stores for the V-type ATPase/antiporter complex present on this membrane, which require considerable amounts of K^+ to maintain rapid fluid production (Wessing & Zierold 1996). In summary, it was proposed that loose binding of ions to the GAG contained within the concretions facilitated the intracellular ion movement necessary for this fluid secretion (Wessing & Zierold 1996). Further evidence of the role played by these intracellular concretions was illustrated by the inhibition of zinc accumulation in concretions in *Drosophila* Malpighian tubule cells by the carbonic anhydrase inhibitors acetazolamide and hydrochlorothiazide (Wessing *et al.* 1997).

The concentrations of elements found in the control treated cells in this study confirm earlier findings. The dense structures contain substantial amounts of many elements such as Na, K and Cl, but no concretions were observed in the lumen of the tubules in either cryofixed or conventionally prepared tubule cells. The high content of some elements in these small areas is alone suggestive of a storage role for the mass dense concretions. The structures pose interesting questions regarding their ability to retain and accumulate K and Na, for example; though their mean concentrations of these elements are comparable with those found in the cytoplasm, what is not clear is, whether like those observed in *Drosophila* (Wessing & Zierold 1996), they are part of the ER/Golgi apparatus endomembrane system. Certainly, the differences in the 3 types' elemental composition does not immediately suggest that they represent 3 forms of the same intracellular structure, due to the substantial differences in the mean concentrations of P, S, Mg and Ca. Clearly, however, the concretions represent a considerable intracellular elemental store, whose function may include the maintenance of fluid secretion. The mass density of these concretions is obviously much greater than that of the cytoplasm, and no estimates have been made regarding the elemental content in mmol l^{-1}, since the

dry weight content will be significantly greater than the figure of 25% used in calculations elsewhere in this study.

Mass dense concretions from control saline plus 1 mM DB-cAMP incubated tubule cells.

The composition and mass density of the concretions identified in DB-cAMP stimulated Malpighian tubule cells altered in comparison with control incubated mass dense concretions (see Table 4.13). The type C concretion was not identified as being present. With regard to the Type A concretion, a substantial fall in the K concentration was observed, and overall the fall in the mass density and the content of P, Ca and especially S suggest that these concretions have also become depleted in their inorganic content as a result of incubation in this stimulant. The K concentration in these concretions was lower than that of the cytoplasm. The fall in K content and the increase in Cl content of the Type A concretion reflect similar changes in the concentration of these elements in the cytoplasm of Malpighian tubule cells incubated in DB-cAMP. Such qualitative changes were not noted in the content and density of the Type B concretions, though the mass density of the bodies did decrease to a greater extent then Type A concretions. S and Mg were the predominant elements in this concretion type, were unaffected by stimulation with DB-cAMP. The fall in the mass density of both Type A and B concretions indicated that there was be a lower amount of elements present in both concretions. Thus, the alterations observed in response to DB-cAMP support the assertion that these structures have some role to play in the ionic economy of *Locusta* Malpighian tubule cells.

Mass dense concretions from control saline plus corpora cardiaca incubated tubule cells.

Corpora cardiaca-stimulated tissue was characterised by the apparent absence from the analysed area of concretions corresponding to the Type A distribution pattern (see Table 4.14). The concretions recognisable as Type B were little different in elemental composition from their control saline incubated counterparts, except that their mean K concentration was raised considerably, to reach a similar concentration to that found in the cytoplasm for this element. However, these concretions were also considerably less mass dense than control Type B bodies. The Type C concretions, the more numerous of the two

types present, increased in K concentration, though Na concentrations fell. The mass density of these concretions was somewhat greater than their equivalent control value. Thus, stimulation with corpora cardiaca extract elicited changes in the mass dense concretions which differed considerably from that of DB-cAMP.

The increase in mass and uptake of Na which was noted in Type C concretions, and K uptake in Type B under C.C. stimulated conditions further supports the possibility of a dual role for the concretions intracellularly, namely as a site of elemental release or uptake, depending on intracellular conditions. The apparent absence of type C concretions as a result DB-cAMP stimulation, yet their predominance in this treatment highlights the possibility that the concretion types might have different roles and variable lability within the cell, depending on the secretory state of the cell. Again, a link between cytoplasmic conditions and concretion elemental content is apparent, since a rise in the concentration of Na intracellularly is reflected in an increase in the Type C content of this element in the C.C. stimulated tubule cells. Uptake of intracellular elements by the concretions was noted by Pivovarova *et al.* (1994a), and this finding supports the idea that the content of the concretions are turned over, with the bodies acting as homeostatic intracellular "buffers" in these cells, as well as possible sites of cation release. The relatively constant concentrations of some elements in the concretions, plus the observation that the concentration of, for example K, matches but does not exceed cytoplasm levels in type C concretions may indicate support for the view that the concretions are relatively inert intracellular ionic buffers. Hence, the content of the concretions has been confirmed as being labile as a consequence of fluid production stimulation by both an exogenous nucleotide and *in vivo* neurohormonal extract.

Chapter Five.

General discussion.

The aim of this final chapter is to consider the results presented in chapters three and four, in light of the currently accepted model of ion transport in *Locusta migratoria* L. Malpighian tubule cells. This model has been discussed in chapters one and three, and is shown in Figure 1.10. Briefly, the basolateral surface is permeable to K^+ but relatively impermeable to Na^+, and possesses both a Na^+-K^+-ATPase (Anstee & Bell 1975; Fogg *et al.* 1991), and an Na^+-K^+-$2Cl^-$ cotransporter (Baldrick *et al.* 1988). Apically, ion transport is driven by a proton gradient generating V-type ATPase (Al-fifi *et al.* 1998b), which powers K^+ and possibly Na^+/nH^+ antiport (Pivovarova *et al.* 1994a). An Na^+/H^+ exchanger has been recently cloned in mosquito Malpighian tubules (Petzel *et al.* 1998). Apical Cl^- channels allow the efflux of this anion down an electrochemical gradient (Baldrick *et al.* 1988). Basolaterally, a K^+ anomalous rectifier may also be present (D. Hyde, pers. comm.).

In the present study, under *in vitro* control experimental conditions, the rate and composition of secreted tubule fluid are similar to values reported elsewhere in this epithelium (Bell 1977; Marshall 1995; Al-fifi 1997). The concentration of K^+ in the tubule fluid is approximately 126mM, that of Na^+, approximately 50mM. The tubule cells possess a gradient in the intracellular distribution of K , and whilst Na and Cl are present, they display no such pattern. The central cytoplasmic K concentration is approximately 116mM. The remainder of the analysed elements, namely S, Mg and Ca, are present at low intracellular concentrations, with the exception of P, whose concentration is the highest of any element, but uniform throughout the cell. The basement membrane displays a pattern of elemental content that reflects the composition of the *in vitro* bathing medium, but as observed in other basal lamina (see Gupta 1989), exhibited an unexpectedly high K concentration. These observations confirm earlier findings in this cell type (Pivovarova *et al.* 1993, 1994a,b) and resemble similar findings in

Rhodnius and larval *Drosophila hydei* Malpighian tubule cells (Gupta *et al.* 1976; Wessing & Zierold 1993).

In this study, no additional work has been performed to further investigate the functioning of the basolateral Na^+-K^+-ATPase. The presence of this protein in *Locusta* Malpighian tubules has been established (Anstee & Bell 1975), it actions explored (Anstee *et al.* 1979; Anstee *et al.* 1986), and its role in the control of intracellular elemental composition measured (Pivovarova *et al.* 1994b). The specific inhibitor of the Na^+-K^+-ATPase, ouabain, has been used to demonstrate the importance of this pump. Ouabain reduces the rate of fluid secretion by *Locusta* tubules (Anstee & Bell 1975; Anstee *et al.* 1979; Donkin & Anstee 1980), and increased the Na/K ratio of the secreted fluid significantly (Anstee *et al.* 1979). This inhibitor also decreased the transepithelial potential (Fathpour *et al.* 1983; Baldrick *et al.* 1988), and depolarised both V_b and V_a gradually (Baldrick *et al.* 1988). The presence of the Na^+-K^+-ATPase on the basolateral membrane in *Locusta* Malpighian tubule cells was confirmed by Fogg *et al.* (1991). X-ray microanalysis showed that ouabain raised intracellular concentrations of Na, and lowered K, but had no effect on Cl (Pivovarova *et al.* 1994b).

In spite of the conclusion that the slow depolarisation of V_b due to inhibition of the Na^+-K^+-ATPase with ouabain indicates that this pump does not significantly contribute to this membrane potential, ouabain slows the rate of fluid production and alters both the composition of the secreted fluid and the intracellular elemental distribution in this epithelial tissue. This study shows that furosemide, the Na^+-K^+-$2Cl^-$ cotransport inhibitor, also slows the rate of fluid secretion *in vitro*, but increased the concentration of Na^+, without affecting the concentration of K^+, in the tubule fluid. The inhibitory action of furosemide on fluid secretion in this tissue has been observed previously; the inhibitor also hyperpolarises V_b, V_a and depolarises of the TEP (Baldrick 1987; Baldrick *et al.* 1988). Results have shown that the addition of furosemide changed considerably the intracellular distribution of elements.

Furosemide abolished the K gradient and lowered the intracellular content of K within the cell. However, both Na and Cl concentrations increased. At the basement membrane, both the concentration of K and Cl was increased. The K^+ concentration in the secreted fluid remains unchanged in spite of a fall in intracellular K concentration. The fall in intracellular K concentration is likely to be

the result of inhibition of this cation's entry across the basolateral membrane in the face of continued efflux across the apical surface. To maintain the level of K^+ measured in the secreted fluid, these tubule cells may also draw on a pool of K^+ separate from the cytoplasm, possibly contained within the mass dense intracellular concretions and available for intracellular release (Pivovarova *et al.* 1994a). The rise in both the concentration of Na intracellularly, and Na^+ in the secreted fluid point to this ion's continued influx across the basolateral surface down its concentration gradient, and retention of this cation intracellularly to maintain a Donnan equilibrium within these cells, since the K content of the cells is reduced. The intracellular increase in Cl concentration at some sites was unexpected. Earlier work indicated that furosemide has a similar electrophysiological effect on V_a to the incubation of *Locusta* Malpighian tubule cells in Cl^--free saline (Baldrick *et al.* 1988). V_a hyperpolarises significantly in both cases, and the decrease in the Cl concentration noted in the apical microvillar region supports the contention that this is a consequence of a reduction in the availability of Cl^- for continued luminal efflux (Baldrick 1987). The increase of K and Cl at the basement membrane may be interpreted as suggesting that basolateral entry of these ions is inhibited by furosemide, and that the accumulation intracellularly of Cl is a result of its presence as a counterion against the increased Na concentration. The maintenance of a similar anion deficit to that seen in control cells supports this interpretation.

In vitro incubation of tubules incubated in Rb-containing saline reduced the rate of secretion in this and earlier investigations (Pivovarova *et al.* 1994a; Marshall 1995). An earlier study using Rb as a K mimic indicated that the concentration of Rb in the secreted fluid remained low, though the tubule fluid remained rich in K^+, and the Na^+ concentration was unchanged (Pivovarova *et al.* 1994a). Intracellularly, this study confirms many of the findings of Pivovarova *et al.* (1994a). Here, the concentration of K fell dramatically, though the gradient seen in control cells was retained. Rb assumed a partial gradient in its distribution, increasing towards the apical microvillar border, and there was a rise in the intracellular concentration of Na apically. Rb did not fully replace K as its concentration was lower than that of K in control incubated cells. In contrast to previous findings, a fall in the intracellular Cl concentration was noted. The anion deficit was reduced within the cell.

These findings are therefore generally consistent with the conclusions of Pivovarova *et al.* (1994a), namely that Na^+ and K^+ exit via separate mechanisms apically, and that Rb^+ cannot compete efficiently at either site; basally, Rb^+ entry must be considerably less efficient than that of K^+ under control conditions. Further, rate of fluid secretion measurements in the presence of both Rb and furosemide performed in this study support the suggestion that the Na^+-K^+-$2Cl^-$ cotransporter is unable to transport Rb^+ (Pivovarova *et al.* 1994a). In combination, furosemide and Rb-saline does not elicit any additional decrease in the rate of fluid production in addition to that of Rb-saline alone; in effect, the cotransporter may be inhibited by Rb-saline. The fall in intracellular Cl noted during incubation with Rb-saline alone supports this interpretation. Rb entry may therefore be primarily via the basolateral Na^+-K^+-ATPase, since Rb may block basolateral K^+ channels in *Locusta* (Marshall 1995) and the hypothesised basolateral anomalous rectifier (Hille 1992; Marshall 1995). On the basis of the relatively unchanged nature of the tubule fluid in response to inhibition by both Rb and furosemide (separately), it is clear that drastic changes in ion transport events at the basolateral surface can be compensated for at the apical membrane. The rise in $[Na^+]$ in the furosemide-inhibited tubule fluid is likely to reflect increased transport of the cation via Na^+/nH^+ antiport as a result of the rise in its intracellular concentration. It is proposed that the proportionally lower rise in intracellular [Na] during Rb inhibition accounts for the unchanged $[Na^+]$ in the tubule fluid (Pivovarova *et al.* 1994a).

Results indicated that bafilomycin A_1, a highly specific V-type ATPase inhibitor, slowed the rate of tubule fluid production, decreased the concentration of $[K^+]$ within the tubule fluid, and raised the concentration of $[Na^+]$. Intracellularly, bafilomycin increased the concentration of K basally, and reduced it apically, though the gradient in this element's distribution was abolished. The K concentration was increased in the basement membrane, but the concentrations of Na and Cl were unchanged. Na concentrations fell within the cell, except at the basal infoldings; Cl concentrations increased, except at the apical microvilli. In both cases, these exceptions are likely to reflect unavoidable microanalysis of the extracellular fluid.

These findings support the presence of an apical V-type ATPase-K^+/nH^+ antiport complex in this epithelium. The build-up of K basally and in the basement membrane suggests that removal of the driving force for apical K

efflux causes an accumulation of K^+ at the basolateral surface, as this cation exits the cell along a concentration gradient, and is recycled across the basolateral membrane via the Na^+-K^+-ATPase. The fall in intracellular Na, and the increase in $[Na^+]$ in the secreted fluid may indicate that basolateral influx of this cation is no longer balanced by apical efflux. This interpretation does not necessarily require a proton gradient-independent apical efflux mechanism for Na^+; since a substantial concentration gradient will favour basolateral K^+ efflux, causing the accumulation of this ion at this surface, and a fall apically. As no such gradient favours Na^+, it is proposed that this cation will instead be "pulled" toward the apical surface, and the residual proton gradient dissipated in Na^+/nH^+ antiport. The increase in intracellular chloride concentrations suggest that Cl^- efflux apically may be reduced, due to the reduction in the overall rate of fluid secretion. The reduction in the anion deficit observed in bafilomycin-inhibited cells may be consequence of the increase in the K concentration within the cells. Abolition of the K gradient and a decrease in K concentration in dry weight terms at the apical surface, and at all cell sites, has been observed in *Drosophila* Malpighian tubule cells when inhibited with bafilomycin A_1; this has been attributed to a loss of water during inhibition (Wessing & Zierold 1993). In wet weights, an increase in concentration was observed basally and centrally, but not apically (Wessing & Zierold 1993).

NEM has also been shown to substantially inhibit fluid secretion in *Locusta* Malpighian tubules (Al-fifi 1997), and increase the intracellular concentration of K (Pivovarova *et al.* 1994b). In particular, no basolateral accumulation of K was noted with this treatment. Na and Cl concentrations were unaffected. The reasons for the difference between these two treatments is not clear. This effect may stem from some NEM inhibition of the basolateral P-type ATPase, preventing the basolateral K^+ cycling thought to account for the rise in this element's concentration basally during inhibition with bafilomycin. A rise in the basal infolding Na concentration may support this. It is not clear why the Cl concentration should remain unchanged under these conditions.

A number of previous studies have reported on the effect of fluid production inhibitors on Malpighian tubules such as those mentioned above, using X-ray microanalysis (Wessing & Zierold 1993; Pivovarova *et al.* 1994a,b). Previous investigations using fluid production stimulators on insect Malpighian tubules were confined to the use of 5-HT in *Rhodnius* (Gupta *et al.* 1976). The

present study has shown that DB-cAMP stimulates fluid production, and alters elemental distribution intracellularly. 1mM DB-cAMP has been shown to stimulate fluid secretion but has no effect on the Na^+ and K^+ concentrations in the secreted tubule fluid (Al-fifi *et al.* 1998a). This study has shown that under stimulation with this compound the intracellular gradient of K was retained, though the concentration of this element was reduced; Na was seen to rise but without a gradient in its distribution. The Cl concentration rose at all cell sites, particularly at the apical regions, though again, no clear gradient was observed. No change was recorded in the concentration of Na, K or Cl in the basement membrane. The mass dense concretions present in these stimulated cells changed in their mass density and elemental content. The Type C, rich in K and Mg, were not found in these cells. The mass density of the remaining Types A and B were considerably reduced; the former decreased in P, Ca and K concentrations but increased their content of Cl.

The increase in intracellular concentration of both Na and Cl would be accounted for if Na^+-K^+-$2Cl^-$ cotransport is activated by cAMP. cAMP has no effect on V_b, strongly suggesting that this cyclic nucleotide acts through a electroneutral mechanism (Fogg *et al.* 1990). Exogenous application of this intracellular second messenger has been shown to stimulate this protein in *Aedes aegypti* (Hegarty *et al.* 1991). The rise in Cl is of particular importance, as the basolateral cotransporter is currently believed to be the chief entry point for this anion basally. An intracellular rise in Cl concentration is suggestive of accelerated entry of Cl^- which is not matched by apical efflux; hyperpolarisation of V_a supports this (Fogg *et al.* 1990).The change in intracellular Cl levels during stimulated fluid production indicates that this anion is transported at least partially transcellularly. The reduction in intracellular K suggests that cAMP-stimulated apical extrusion of K^+ is not balanced fully by entry of this cation basolaterally. The incomplete nature of stimulation with cAMP alone, in comparison with crude corpora cardiaca extract, has been noted previously (Al-fifi *et al.* 1998a). The fall in intracellular K may be interpreted as supporting the hypothesis that the second site of basal K^+ entry, the Na^+-K^+-ATPase, may require activation by another second messenger system (Al-fifi *et al.* 1998a). Direct stimulation of the apical V-type ATPase by cAMP, as suggested by Al-fifi *et al.* (1998a) may steepen the proton gradient across the apical membrane, resulting in an equivalent increase in both Na^+ and K^+ antiport mediated efflux

without changing the Na^+ / K^+ ratio of the secreted fluid, and causing the observed intracellular depletion of K to occur (Al-fifi *et al.* 1998a). Rp-cAMP, the protein kinase A inhibitor, lowers the tubule fluid secretion rate, it is suggested by inhibition of both the apical V-type ATPase and the basolateral Na^+-K^+-$2Cl^-$ cotransporter (Al-fifi *et al.* 1998a).

Evidence is available to confirm the lability of the mass dense intracellular concretions in DB-cAMP stimulated cells; one type selectively loses K, and gains Cl, both become less mass dense. This supports the contention that these structures act as ion stores (Pivovarova *et al.* 1994a), since the changes reflect qualitatively the alterations occurring in the cytoplasm of these cells. K^+ may be released from these structures in response to falling cytoplasmic levels of this cation; conversely, Cl^- may be taken up to buffer the cell against excessive osmotic stress. Such a mechanism is conceivable in Malpighian tubule cells, which must tolerate very rapid rates of turnover of their ion and fluid content. These changes in the content of the mass dense concretion also point to a relatively passive mechanism for uptake or release; it may be speculated that the ionic binding capacity of these structure is merely a consequence of the nature of their chemical structure, and that no active (i.e. pump-driven) system occurs in the concretions. This assertion is supported by the model of Wessing and Zierold in *Drosophila* (Wessing & Zierold 1996).

The second fluid production stimulator used in these cells, corpora cardiaca has a qualitatively and quantitatively different effect on fluid production to that of cAMP, and additionally, as will be shown, on the elemental composition of the cell. Crude corpora cardiaca extract stimulated fluid production to a greater extent than cAMP in this and previous studies (Al-fifi *et al.* 1998a), and significantly in comparison with cAMP, altered the ratio K^+ and Na^+ in the secreted fluid. The secretion of Na^+ was selectively stimulated to a greater extent than that of K^+ (Al-fifi *et al.* 1998a). Intracellularly, results from this study demonstrate that this neurohormone altered only the distribution and concentration of Na^+. K retained its gradient and was present at largely similar concentrations, and Cl concentrations were also predominantly equivalent to the control cell values. The concentrations of Na, K and Cl were all greater in the basement membrane than those values seen in controls. It may be that this reflects an additional cycling of Na^+ and K^+ across the basolateral membrane; the rise in intracellular Na^+ concentrations may be counteracted by an increase

in Na^+-K^+-ATPase activity, and causing the observed increase in the K concentration at the basal infoldings. The reduction in the intracellular concentration of Cl in comparison with cAMP stimulation supports the suggestion that an additional effect of corpora cardiaca extract is to stimulate anion efflux apically. This was proposed from the depolarisation of V_a observed on corpora cardiaca stimulation, whereas cAMP caused a hyperpolarisation (Fogg *et al.* 1990). Corpora cardiaca extract was seen to elicit an increase in intracellular levels of both cAMP and IP_3 in *Locusta migratoria* Malpighian tubules (Fogg *et al.* 1990). It is suggested that the rise in intracellular K and the fall in Cl seen during stimulation with corpora cardiaca extract, in comparison with stimulation using cAMP, are caused through the action of the IP_3 / PKC second messenger pathway. Stimulation of vertebrate nephron Na^+-K^+-ATPase via the PKC pathway has been demonstrated previously (Chen *et al.* 1993). At the apical surface, the preferential increase in the rate of extrusion of Na^+ during stimulation with corpora cardiaca extract (Al-fifi *et al.* 1998a) is suggestive of a direct stimulatory effect on Na^+/nH^+ antiport via the PKC pathway. The increase in intracellular Na concentration observed by X-ray microanalysis in this study, particularly in the apical region of the cell, would support the rise in tubule fluid [Na^+].

The lability of intracellular mass dense concretions during stimulation with cAMP was complemented by the changes observed during stimulation with corpora cardiaca extract. The observed rise in the concentration of K in the type B concretions probably reflects an increasing intracellular turnover of this element in this secretory state. No type A concretions were recorded from these cells. These findings, and those discussed in relation to the changes in concretions during DB-cAMP prompt speculation as to the function of these enigmatic structures. Certainly, the appearance of concretions enriched in the two major cations of the tubule fluid, during corpora cardiaca stimulated fluid production supports the model advanced in *Drosophila*, in which the concretions are primarily viewed as a K^+ delivery mechanism supporting apical ionic transport (Wessing & Zierold 1996). The ability of the concretions to accumulate Cl and lose K, under quite different stimulatory conditions, perhaps points to a more general interpretation of the role of these bodies, namely that they are primarily intracellular homeostatic buffers, responding passively to perturbations in the intracellular ionic environment. Such a mechanism would be of considerable

advantage to these epithelial cells, since their rate of flux of ions and water is well known to be prodigious (Maddrell 1991).

Finally, some consideration must be given to the nature of the gradient of concentration of K seen in these cells, in this and earlier studies in *Locusta migratoria* Malpighian tubule cells (Pivovarova *et al.* 1994a,b). Results obtained in this study provide support for the conclusion that this gradient, and fluid secretion are intimately linked. Inhibition of fluid production by furosemide and bafilomycin is accompanied by the dissipation of the concentration gradient, whilst stimulation with DB-cAMP and corpora cardiaca extract is characterised by a retention of the gradient, though its magnitude is reduced in the case of the former stimulant. It may be, that as suggested by Wessing and Zierold (1996) in *Drosophila* larval Malpighian tubules, the gradient develops as a result of the movement of K^+-binding mass dense concretions from the ER-Golgi complex to the apical surface, where K^+ is unloaded and the GAG content of the Golgi derived vesicle endocytosed, to later coalesce into PG-rich mass dense concretions in the lumen. However, no mass dense concretions have been observed in the lumen of the *Locusta* Malpighian tubules in this or previous studies (Bell & Anstee 1977); moreover, gradients of Na^+ and K^+ have been observed in epithelial cells not characterised by the presence of these concretions intracellularly, such as toad urinary bladder (Rick & Schratt 1989). In this tissue, the increasing apical to basal gradients of both Na and Cl during ADH-induced osmotic water flow have been proposed to be a consequence of a non-uniform distribution of fixed negative charges, caused by water flow (Rick & Schratt 1989). This may be the case in *Locusta* Malpighian tubules, though unlike toad urinary bladder, no gradient of P accompanies that of K, which serves in the latter epithelia to provide the fixed negative charge gradient (Rick & Schratt 1989). It is axiomatic that the transport of active transport of K^+ from haemolymph to lumen in *Locusta* Malpighian tubules facilitates water flow and hence fluid production (Bradley 1985). In light of the changes in intracellular K concentration during inhibition with bafilomycin A_1, it is tentatively proposed that the increasing gradient in K intracellularly is due to the action of the V-type ATPase / K^+/nH^+ antiport complex. The higher concentration of K apically, and its high content in the tubule fluid, suggests that the V-type ATPase / K^+/nH^+ antiport complex somehow "drags" K across the cells. Further work is clearly needed on this unusual aspect of ionic distribution in these epithelial cells.

In conclusion, the data presented here are consistent with the current model of ionic transport in *Locusta migratoria* L. Malpighian tubules (Figure 1.10). This study supports a dynamic role for the mass dense concretions in K handling in these cells. Furthermore, the work emphasises the integrated nature of transport protein activity during stimulated secretion. This integration is likely to be achieved through the activation of these proteins by intracellular second messengers at both apical and basolateral membranes; an activation that is likely to be a consequence of covalent modulation via protein kinase and phospolipase activity (Al-fifi *et al.* 1998a).

Future work.

Building on the work described in this thesis, future investigations into *Locusta* Malpighian tubules could include the use of thapsigargin and/or ionomycin, to study the Ca^{2+}-mediated arm of fluid production. These compounds have been applied to *Acheta domesticus* Malpighian tubules (Coast *et al.* 1993), and in conjunction with the integrated experimental approach used here, could be used to similarly probe *Locusta*. The stimulatory response of *Acheta* tubules to achetakinins is mimicked by these 2 compounds. Achetakinins, as members of the kinin family of neuropeptides, share a highly conserved C-terminal pentapeptide core sequence with leucokinins. Application of achetakinins may provide additional information pertaining to the nature of Cl^- transport in *Locusta migratoria* type 1 Malpighian tubule cells, and the status of a possible paracellular Cl^- shunt.

Of further interest is the recent observation that in the upper tubule region *Rhodnius*, exogenous cGMP is antagonistic to cAMP (Quinlan & O' Donnell 1998). cGMP is a potent antidiuretic in this tissue, and it may be that it acts through a cAMP-degrading phosphodiesterase (Quinlan & O' Donnell 1998). Again, this experiment could be simply replicated in *Locusta*. The presence of such an antidiuretic mechanism in *Locusta* would be of particular interest, in light of the considerable differences in the functioning of the tubules in comparison with *Rhodnius*.

However, the nature of elemental accumulation with the cytoplasmic mass dense concretions in the tubule cells remains unclear. This study, and other work (Wessing & Zierold 1996) suggest that they are involved in the ionic

economy of Malpighian tubule cells. The uptake of K and other elements into the concretions could be quantified by the use of radiolabelled isotopes of these elements, introduced into the cells through the bathing media. Fractionation of the cells and the isolation of the concretions could be achieved by density gradient centrifugation, for example. Intact concretions have been observed by electron microscopy in pellets obtained from a recognised membrane preparation protocol used in this tissue (author's unpublished observation) (Al-fifi *et al.* 1998b). Isolation and purification of the concretions would provide a starting point for studies on the dynamics of elemental loading and unloading in these enigmatic structures, and provide further information on the possible role(s) of these bodies.

Appendix One.

Biological X-ray Microanalysis.

X-ray microanalysis is a chemical analytical procedure which permits the measurement of the elemental content of small areas of unknown specimens, as a consequence the emission of X-ray photons. The spatial resolution of analysis can theoretically be as low as 10-20nm (Hall 1971; Warley 1997), though a range of 100-200nm is more realistic in biological microanalysis (Hall & Gupta 1983). Two forms of X-ray microanalysis exist, wavelength dispersive spectrometry (WDS) and energy dispersive spectrometry (EDS) (Hall & Gupta 1983; Warley 1997). This appendix will primarily consider the latter, since it is currently used in the majority of biological investigations, including this study.

Wavelength dispersive spectrometry is dependant upon the use of diffracting crystals, orientated at a specific angle so that emitted X-ray photons are reflected onto a detector, often a gas flow proportional counter (Hall 1971). The diffractor is extremely specific for the particular characteristic wavelength to which is orientated, but by definition, WDS can only identify a single element at a time (Chandler 1977). Though the gas flow counter can be used as a "stand-alone" elemental detection system to identify multiple X-ray energies simultaneously, its resolution is limited; additionally, the efficiency of WDS is low (Chandler 1977; Hall & Gupta 1983).

X-ray photon detection is effected in energy dispersive spectroscopy by a solid state detector, either an Si(Li) (silicon-lithium doped, or "drifted") or HpGe type (high purity germanium) (Chandler 1977; Blok-van Hoek & Pinxter 1993). The energy resolution of such detectors is inferior to that of the combined diffracting crystal/gas flow counter used in WDS, but for thin sections of biological material, the solid state detector is simpler to operate and may be positioned closer to the specimen in order to maximise sensitivity (Chandler 1977). Table 1 briefly summarises the advantages and disadvantages of these two types of microanalysis.

Table 1. The advantages and disadvantages of energy dispersive and wavelength dispersive spectrometry (adapted from Chandler, 1977).

Energy dispersive spectrometry (solid state detector)	Wavelength dispersive spectrometry (diffracting crystal/gas flow counter)
Advantages.	**Advantages.**
1. High count rate detectability allows small probe diameter, reducing specimen damage.	1. Good peak-to-background ratio, high sensitivity to trace elements.
2. Easily retrofitted to EM.	2. Resolving power of X-ray lines high.
3. Computer compatible output.	
4. Detector efficiency approaches 100% for Z ≥18.	3. Sensitive to light elements (Z > 8).
5. Simple mechanical design	
6. Spectra displays all elements, enabling rapid quantitation.	4. Small dead-times during quantitation.
Disadvantages.	**Disadvantages.**
1. Inferior energy resolution in comparison with WDS.	1. Analysis only possible for one element at a time.
2. Low quantitation accuracy at low elemental concentrations.	2. Possible errors introduced due to mechanical system.
3. Extensive background corrections required.	3. Separate quantification of peak and background necessary.
4. Light element sensitivity often low due to X-ray absorption by detector window.	

The ability of EDS to achieve high count rates under conditions which minimise specimen damage is of particular importance when analysing biological material, and this form of microanalysis now predominates biological investigations (Hall & Gupta 1983). The high beam currents needed for WDS can cause substantial specimen damage (Morgan 1985). However, WDS continues to used in studies on insect secretory epithelia, for example, in the elemental analysis of the fluid secreted by Malpighian tubules (Williams & Beyenbach 1983; Pannabecker *et al.* 1993). For further information on WDS, see Reed (1993).

The theoretical basis of energy dispersive X-ray microanalysis.

The Bohr atomic model proposes that an atom consists of a positively charged nucleus, surrounded by a series of concentric shells, or orbitals, containing negatively charged electrons. The energy of the electrons increases with distance from the nucleus, and therefore varies depending upon which atomic orbital it occupies (Morgan 1985; Warley 1997). Crucially, the potential energy of the orbital electrons also depends upon the net positive charge on the nucleus, that is, on the atomic number of the element (Morgan 1985; Warley 1997). If an incident radiative source, for example a stream of electrons, bombards an atom, a number of interactions result, see Figure 1 (Morgan 1985). The majority of incident electrons interact with weakly bound outer shell electrons and undergo small-angle or elastic scattering with some dissipation of their energy (Marshall 1980; Morgan 1985). If an incident electron passes close to the nucleus of the atom, it may interact with the electrostatic field of the nucleus, and in doing so undergo a more substantial change in its angular direction, decelerate, and may lose energy in the form of an X-ray photon (Marshall 1980; Warley 1997). The radiation thus produced is commonly known as continuum radiation, theoretically with an energy distribution from infinity to that of the energy of the incident electron (Marshall 1980). Continuum radiation is consequently produced by inelastic scattering of incident electrons by the atomic nucleus; an inelastic collision between an orbital electron and an incident electron may cause two different events to occur. The incident electron may undergo a smaller change in angular direction than in elastic interactions and loses energy; firstly, an outer orbital or "bound" electron may be ejected from the atom entirely (an Auger electron), or secondly, an inner shell

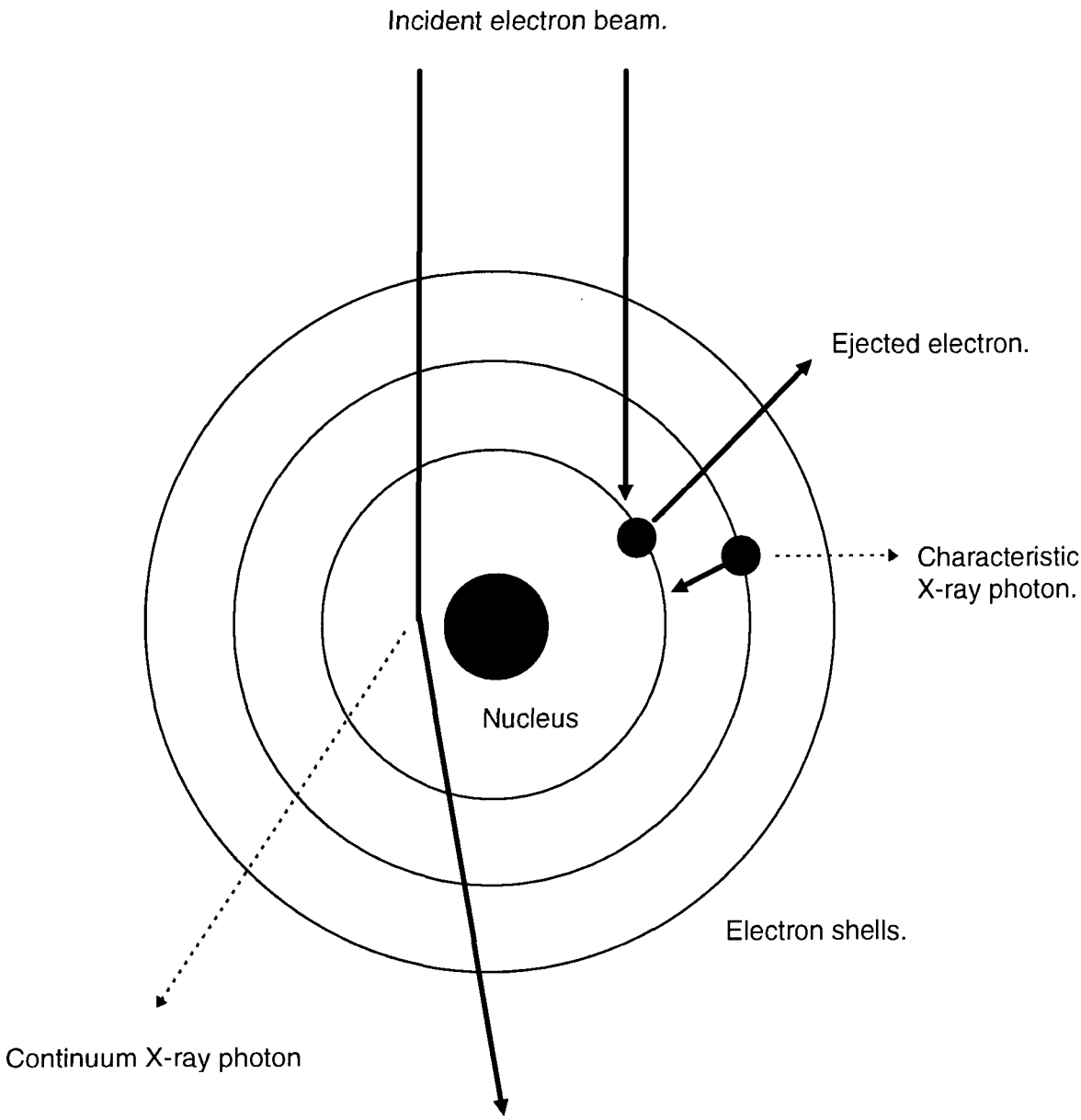

Figure 1. Ionisation events during incident electron beam irradiation of an atom (adapted from Warley, 1997). The production of both characteristic and continuum X-rays as a result of inelastic scattering is shown. Elastic scattering events are not illustrated. The interaction on the left depicts an inelastic scattering event as an incident electron passes close to the atomic nucleus , and the result is the emission of a continuum X-ray photon. On the right, an inelastic collision between an incident electron and an inner shell electron causes the emission of a characteristic X-ray photon.

electron may be ejected from its normal energy level (Morgan 1985; Warley 1997). Should an electron be removed from an inner shell, an outer shell electron from that atom will drop into gap created in the lower energy inner shell (Warley 1997). Since an electron drops from a higher energy shell to a lower energy shell, the difference in energy is emitted as an X-ray photon (Agar *et al.* 1974; Warley 1997). The exact energy of the X-ray photon is dependant upon the difference in the energy between the two electron shells, and is unique to a specific atom since this difference varies with the charge on the nucleus, i.e. the atomic number of the element (Agar *et al.* 1974; Warley 1997). The X-ray is said to be characteristic to the element from whose atom it is emitted, as its energy varies simply with the atomic number of the element (Warley 1997). This forms the central tenet of energy dispersive X-ray microanalysis: when an atom of an element is irradiated by an incident electron beam, an emission of X-rays occurs whose energy contains information relating to the atom from which the X-ray is emitted. It must be stressed that X-ray microanalysis consequently provides information regarding elemental distribution alone, and not the state of the element, that is, either in a bound or in a free ionic state.

In practice, the production of X-rays is neither a single discrete event, nor as simple as the preceding paragraph suggests. Displacement of an electron from a shell normally initiates the movement of a number of electrons, which cascade into lower energy shells and generate a number of X-rays of different energies (Warley 1997; Morgan 1985). In this way, incident irradiation of an atom causes the production of a family of X-rays whose pattern is dependant upon the atomic number of the element (Warley 1997). This pattern is usually displayed in the form of an X-ray spectra, which is a frequency plot of the number of X-ray quanta measured at each shell energy level (Warley 1997). The peaks in such spectra are variously described as, for example, potassium Kα (K Kα) or K Kβ, terms which define the exact energy levels from which electrons originated from, and dropped into, to generate X-rays from this element with the energies shown on the abissca axis. K Kα, for example, describes the peak around the energy level of X-rays produced when electrons in the potassium L shell drop in the K shell (Chandler 1977; Warley 1997). Characteristic peaks are produced in the spectra rather than discrete single lines as a consequence of widening of the X-ray signal during detection and processing. The peaks are found above a

background derived from the continuum radiation produced by irradiation of the specimen (Chandler 1977; Warley 1997).

However, in order for the incident electron to initiate the emission of an X-ray from an atom, the energy of the electron (E_O) must exceed the critical excitation potential (E_C) of that atom. That is, is must have sufficient energy to remove an inner shell electron from the atom of the element under analysis (Chandler 1977; Morgan 1985; Warley 1997). Optimal efficiency is achieved when $E_O \geq 2.7E_C$ (Chandler 1977). The practical implication of this is that if analysis is carried out below certain probe voltages, certain heavy element spectral lines will be unable to be detected. In this study, a constant probe voltage of 100 kV was used (Chandler 1977). Difficulties usually only arise at the low probe voltages used in scanning electron microscopy. Assuming $E_O > E_C$, the probability that an incident electron will ionise an atom is given by the ionisation cross-section, Q, (Coslett and Green 1961) where;

1. $$Q = (1 / E_O E_C) \log_E (E_O / E_C).$$

Assuming that ionisation occurs, the X-ray photon may not be emitted as a result of secondary interactions (Chandler 1977). The term fluorescent yield describes the probability that an X-ray will be produced by the incident electron interaction, and its magnitude is governed by the atomic number of the element (Chandler 1977; Warley 1997). The production of an X-ray photon does not guarantee that it will exit the surface of the specimen and thus be available for detection, due to absorption by the specimen. The magnitude of this X-ray absorption depends on the thickness and composition of the specimen, and also the energy of the X-ray, and the distance through the specimen that X-ray photons must traverse before leaving its surface (Chandler 1977). Reabsorption of the photon may instead cause the production of an Auger electron (Burhop 1952). Absorption may also occur in the detector window or parts of the interior of the electron microscope. Additionally, X-ray photons generated by primary ionisation events can cause secondary ionisation of other specimen atoms, if the X-ray photon energy is high enough. This is called secondary X-ray fluorescence (Warley 1997). Further details regarding the complex nature of X-ray production during X-ray microanalysis may be found in Chandler (1977), Morgan (1985) and

Warley (1997), or in review articles by Marshall (1980) or Blok-van Hoek and Pixnter (1993).

Practical aspects of energy dispersive X-ray microanalysis.

Early WDS microanalysers consisted of a separate electron gun and optical microscope, but since the combination of a diffracting spectrometer and an SEM (Duncumb 1959), X-ray microanalysis has usually been carried out in conjunction with the high resolving power of the electron microscope (Warley 1997). A transmission electron microscope has been used in this study, but a scanning microscope may also used in STEM mode for X-ray microanalysis (e.g. Zierold 1988; Warley *et al.* 1983). In either case, the source of incident electrons is the microscope electron beam. Use of the electron microscope has the advantage of enabling analysis to be carried out with good ultrastructural resolution, since the electron beam may be focused upon visible microareas in the specimen.

The X-ray detector system usually consists of a Li-drifted Si semi-conductor crystal, held under a vacuum and cooled in liquid nitrogen (Chandler 1977; Warley 1997). X-rays pass into the detector through a thin beryllium window, about 8μm thick (Morgan 1985; Warley 1997); windowless detectors are rarely used in biological microanalysis (Warley 1997). The X-rays impact the silicon atoms in the semi-conductor and create electron-hole pairs by ionisation, the number of which is proportional to the energy of the X-ray (Chandler 1977; Warley 1997). A large potential difference is maintained across the crystal, which causes the electrons and "holes" to migrate in opposite directions; hence, a pulse of current is generated for each hole pair formed (Morgan 1985). Though not all the X-ray energy is converted into electron hole pairs, the pulse of current produced varies with the X-ray energy, and is subsequently passed to a field effect transistor (FET) / pre-amplifier circuit (Chandler 1977; Warley 1997). This circuit produces a voltage output with a step-stair pattern, each step representing the arrival of a single X-ray photon on the detector, and its height being proportional to the energy of that X-ray (Warley 1997). The output then passes to a pulse processor unit (PPU) which converts the stepped output to discrete voltage pulses, and filters the output to reject pulses generated by X-ray photons which arrive simultaneously at the detector (pulse pile-up) (Warley 1997). The

voltage output from the PPU is modified to a digital signal via an analogue-to digital converter, and passes to a multichannel analyser where each signal is sorted into its appropriate energy channel (Morgan 1985). The number of signals per channel are counted over a given period and plotted as a histogram, with the X-ray energy as abissca axis, and the ordinate axis as the number of counts or X-ray intensity (Warley 1997). The spectra are usually displayed on a screen, or additionally in the form of a print-out. Modern systems routinely include a microcomputer which enables rapid identification and quantification of the elements present in the specimen. Solid state detectors must be cooled with liquid nitrogen to minimise system noise and to limit the mobility of Li atoms in the semi-conductor crystal, and the maintenance of a vacuum around the crystal assists with this cooling (Warley 1997).

The X-ray spectrum derived from the specimen after processing differs in some important aspects from the actual spectrum emitted from the specimen. The quantum efficiency of the detector is low for both high and low ($\leq$ 2 keV) energy X-rays, in the latter case because light element ($Z < 11$) X-ray photons are partly absorbed by the beryllium detector window (Morgan 1985). The fact that detection efficiency varies, reaching a maximum when $Z > 15$-18 is used in the calibration procedure described in section 4.2.6 of chapter 4 (Warley 1990; Warley 1997). As a consequence of noise in the detector and processor system, and variations in the charge produced by electron-hole pair migration, characteristic peak broadening occurs. This broadening may cause overlap of adjacent peaks, which may confuse elemental identification and quantification, though most analysis programs now deconvolute such overlaps (Warley 1997). The broadening of a characteristic peak is termed the resolution of the said peak, defined as the full width at half the maximum height of the peak (FWHM) and equal to;

3. $$FWHM = \sqrt{5.52\, F_{\varepsilon}\, E_O}.$$

where $F\varepsilon$ = the Fano factor, a correction constant specific to the semi-conductor type used in the detector. Additional sources of errors are introduced by excessively large dead times, due to large input count rates, spectral artefacts such as low energy tailing due to incomplete charge collection by the detector, and the production of elemental escape peaks because of characteristic X-ray

production in the detector semi-conductor itself (Warley 1997). Dead-time refers to the period during which the detector is unable to register X-ray photon arrival, due to the periodic resetting of the FET / pre-amplifier circuit, and as a consequence of pulse pile-up filtering by the PPU (Morgan 1985).

Specimen preparation.

Elder and co-workers (Elder *et al.* 1997) have compared some of the difficulties faced by biologists and material scientists in the preparation of suitable thin sections for microanalysis. As X-ray microanalysis of a specimen ordinarily requires its introduction into the vacuum of an electron microscope, biological material must be stabilised in a non-aqueous form prior to analysis. Though limited use has been made of X-ray microanalysis in a biological context in the environmental scanning electron microscope (ESEM) (Sigee 1997), analysis of fixed specimens remains the norm. Furthermore, if useful data regarding the spatial distribution of elements is required, then the specimen must be transparent to the incident electron beam. The preparation of tissue prior to analysis is of unrivalled importance, since no detection system or calibration protocol can compensate for poor fixation or damaged sections (Sigee *et al.* 1993).

A large and comprehensive literature exists regarding biological specimen preparation for EDS (for example see Chandler, 1977; Hall 1979; Somlyo and Silcox 1979; Morgan 1980; Zierold 1988, 1990, 1993; Warley 1997). X-ray microanalysis has also been carried out on microdroplets of biological fluid (Lechne & Warner 1979; Quamme 1988; Roinel 1988; Morgan & Winters 1993; Roinel & Rouffignac 1993) but the procedure will not be considered further here. Similarly, it is beyond the scope of this section to consider non-cryogen based fixation techniques which have been used in some studies to prepare some tissue for quantitative biological X-ray microanalysis, for example precipitation (Somlyo 1985).

The nature of the tissue preparative technique used depends not only on the form analysis takes, but on the information that is required from the tissue during analysis. The aim in this study was to obtain quantitative intracellular chemical composition data from subcellular regions of *Locusta* Malpighian tubules with reasonable spatial resolution. The most suitable fixation process

was therefore rapid cryofixation, ideally using an inert cryogen such as Arcton 22, Freon 12 or liquid nitrogen (Warley 1997). Cryogens used in other investigations include ethane and propane; a range of different methods such as plunge freezing, jet freezing, impact freezing and high pressure freezing can be employed to achieve good cryopreservation with an optimal cryofixation temperature of -135°C or less (Zierold 1993; Warley 1997). For X-ray microanalysis, cryofixation has an number of important advantages over conventional fixation (Hall 1979). It allows rapid fixation, in the order of 10^4 K s^{-1}, enabling the cells' internal processes to be more or less immediately stopped on fixation and additionally minimises the diffusion of ions intracellularly (Zierold 1993). Avoiding the use of chemical fixatives and dehydrating agents also assists in the retention of the *in vivo* intracellular ionic content and its subcellular location (Warley 1997). High molecular mass cryoprotectants such as dextran are sometimes used in studies where retention of the ionic constituents of aqueous spaces are is necessary (Warley 1997). Nevertheless, however good the cryofixation procedure is, adequate cryopreservation is usually restricted to the uppermost 10μm or so of the cryofixed block (Morgan 1985). In this region, the tissue water becomes vitrified, that is, it solidifies without ice crystal formation (Warley 1997). Below this is a thin layer in which small cubic ice crystals form which are not large enough to disrupt X-ray microanalysis, followed by layers where the lowered thermal conductivity of the frozen regions have slowed the rate of fixation so that large hexagonal water crystals form (Warley 1997). The importance of rapid quench freezing of specimens has been emphasised by Morgan (1985), since it reduces the size of the small cubic ice crystals which are inevitably formed in the specimen, and assists in reducing the risk of sample contamination. Small cubic ice crystals do not unduly limit microanalysis as long as they are smaller than the microarea routinely analysed (Morgan 1985).

Once the biological tissue has been cryofixed, it is necessary to cut the material into thin sections for analysis in the transmission electron microscope. To prevent further propagation of ice crystals and the attendant displacement of elements, the frozen tissue must be kept at cryogenic temperatures throughout the sectioning process, usually in the range -120 to -130°C (Warley 1997). Cryofixed blocks of tissue may already be frozen onto specimen pins, or "glued" to a pin after fixation with a cryogen, e.g. heptane (Warley 1997). Sectioning is carried out using dry glass knives with varying cutting speeds, knife angles and

knife and specimen temperatures depending upon the type of tissue being sectioned. The thickness to which the sections are cut also varies, but can be divided into either thin (0.5-2μm) of ultrathin (50-200nm), depending upon the type of analysis to be carried out (Hall 1986). Routine cryoultramicrotomy of freshly frozen biological material is difficult, but good quality sections of cryofixed cells can be regularly obtained with current equipment (Parsons *et al.* 1984; Morgan 1985; Warley 1997). Sections of thickness 1μm or greater are usually produced during studies in which analysis is carried out on the hydrated section without further stabilisation (Zierold 1990). Examples include *Calliphora* salivary gland and rectal papillae, rabbit ileum, anterior caeca of *Schistocerca* and *Manduca sexta* midgut (Gupta *et al.* 1978; Gupta *et al.* 1978, 1979, 1980; Dow *et al.* 1981, 1984). Obtaining adequate contrast is a problem in such sections (Gupta 1979; Gupta & Hall 1982; Dow *et al.* 1984; Zierold 1985). After analysis, the hydrated sections are usually dehydrated within the EM microscope column then analysed in the dried state, to enable subsequent calculation of the elemental concentrations in mmol Kg^{-1} wet weight (Gupta 1979; Dow *et al.* 1984; Warley and Gupta 1991; Warley 1997). Though this produces data in the form of more physiologically relevant units, sectioned material is more commonly frozen dried under a vacuum prior to analysis, leading to results in form of concentration per unit dry mass of specimen (mmol Kg^{-1} dry weight) (Somlyo & Silcox 1979; Zierold 1990). Once sectioned, the small frozen sections are generally transferred from the surface of the glass knife to a plastic coated grid, with a large mesh size or of the parallel bar type (Zierold 1990; Pivovarova *et al.* 1994a,b) using a clean, cooled eyelash probe (Zierold 1990; Warley and Gupta 1991). The sections are then flattened using a second grid and cooled metal bar (Zierold 1990); it should be emphasised that this procedure is carried out within the cryoultramicrotome chamber to prevent section rehydration (Warley 1997). Freeze-drying may be carried out in the electron microscope column, or more usually overnight in a specialised freeze-drier unit with an Peltier adjustable temperature stage (Steinbrecht & Müller 1987; Warley 1997). Once stabilised in this way, the sections are often carbon coated to prevent rehydration during handling and analysis of the grids, to assist in protection of the sections from beam damage during analysis, and to reduce contamination of the sections in the electron microscope column (Chandler 1977; Warley 1997). Cooling of the specimen during analysis also helps to reduce beam damage (Hall & Gupta

1974), since even with probe currents as low as 0.1 to 1.0 nA the critical dose of energy per unit area for damage to an uncooled frozen dried section may easily be exceeded (10^{-10} C μm^{-2}) (Hall & Gupta 1974). The frozen dried sections produced are therefore in a form in which both the cellular ultrastructure and elemental distribution is preserved for analysis.

Quantification.

To identify accurately the presence and amount of an element in an unknown specimen, the detector system must be calibrated, in a manner appropriate to the type of sample to be analysed and the detector system in operation. The positions of the characteristic peak of all the elements expected to be encountered must be checked to ensure they are in the correct energy channel, and the correct shapes of these peaks confirmed (Warley 1997). The detector resolution, i.e. FWHM of a selected element peak, may also be measured. Quantitative analysis usually requires the preparation of standards which mimic the tissue to be analysed but contain a known amount of key element, and solutions made from purified proteins such as bovine serum albumen (Dörge *et al.* 1974) and gelatin (Roomans & Séveus 1977) are particularly appropriate for analyses of frozen-dried biological material (Warley 1990, 1997), though aminoplastic resins may also be used (Roos & Morgan 1985). Protein-based solutions thus act as synthetic "cells". It is not necessary to prepare such standards for each element to be studied (Morgan 1985). Morgan *et al.* (1975) introduced the relative detector efficiency method, which relies on variations in detector efficiency resulting from variations in the energy of emitted X-ray photons of different elements. In some instances, peripheral standards have been used to confirm elemental concentrations. These take the form of protein or dextran-based solutions with known elemental compositions, and tissue to be analysed are dipped in them prior to cryofixation and sectioning (Rick *et al.* 1979; Warley 1997).

The theoretical basis of the quantification method used in this study is described in some detail in the following pages. In essence, qualitative analysis relies on the energy channel position of the specific characteristic peaks in the X-ray spectra, whilst quantitation relies on the intensity of the characteristic X-ray (i.e. the number of counts per channel) being proportional to the amount of that

element present in the microarea analysed (Morgan 1985). Since the intensity of continuum radiation produced from a specimen is directly proportional to the mass per unit area of the microarea analysed, the amount of the unknown element is derived in the form of a concentration per total mass of specimen analysed in that microarea (Warley 1997). The basis of this continuum method is described in additional detail in papers by Hall and co-workers (Hall 1971,1979; Hall & Gupta 1983)

Other quantification methods for thin sections are available, for example the peak intensity method (Rick *et al.* 1978). A comparison between these two types of quantitation suggested that the use of peripheral standards in the peak intensity method altered the composition of certain cell types in rat kidney (Saubermann *et al.* 1988). The region over which the continuum is measured varies, but deconvolution subroutines now available make it unnecessary to avoid regions containing characteristic peaks (Warley 1997). Since extraneous sources of background radiation contribute to the continuum and characteristic radiation, corrections must however be made to compensate for the generation of X-rays by the grid, support film and grid holder (Hall 1979).

The theoretical basis of continuum-normalisation.

Using Hall's terminology (Hall 1971) thick specimens describes sections an incident electron cannot pass through to exit via the far surface (i.e. are greater than 10μm in thickness). Thin (0.25 to 5μm) and ultrathin sections (200nm or less) are thin enough to permit electrons to pass through without losing a substantial proportion of their energy, though it is clear that electron transmission depends on probe voltage (Hall 1971). In this study, the frozen-dried sections analysed were likely to have been predominantly ultrathin according to these criteria, due to an overall likely shrinkage of some 10-20% during freeze drying (Zierold 1988). Expressed in mass per unit area, this equates to a decrease of some 20-25% (T. A. Hall, pers. comm.). Continuum normalisation was developed primarily for thin (1μm) sections, since at that time the sectioning of tissue at a thickness of 300 to 200 nm and at the low temperatures required to retain satisfactory *in vivo* elemental distribution had not been perfected (Hall 1971). The theory is nonetheless valid for both thin and ultrathin sections (Hall 1971). In the case of the latter section type, with probe

voltages in excess of 30kV, incident electrons pass essentially unchecked through the specimen and the spatial resolution on the section is equivalent to the probe diameter (Hall 1971).

Elemental identification from emitted X-ray quanta with wavelength dispersive spectrometry is possible since;

3. $1 / \lambda = k (Z-z)^2$ (Moseley 1913)

where;

λ = wavelength of excited X-ray line.

k = a constant which depends upon the spectral series analysed.

Z = atomic number.

z = screening constant.

But since;

4. $E = hv = hc / \lambda$

where;

E = quantum energy.

h = Planck's constant.

c = the speed of light.

λ = wavelength.

v = frequency.

Thus,

5. $E = k (Z-z)^2$ (Warley 1997)

It is therefore possible to infer the atomic number and hence identify the element from the energy of its emitted X-ray quanta when it is irradiated by an incident electron beam. This is the basis of energy-dispersive X-ray microanalysis.

As an incident electron passes through a specimen, it travels a distance equal to *ds* and for an element *x,* generates an average number of characteristic quanta equal to $\eta_X ds$, where;

6. $\eta_X ds = w_X q_X N_X ds$ (Hall 1971)

where;

w_X = fluorescent yield for element x.

q_X = ionisation cross-section for element x.

N_X = number of atoms of element x cm^{-2}.

ds = a small part of the trajectory of an incident electron.

At the same time, the electron also generates an average number of quanta equal to $\eta_b\ dV\ ds$, this being the continuum (bremsstrahling) quanta, generated in the range V to (V + *dV*) when;

7. $\eta_b\ dV\ ds = (K/E)(dV/V)\ \Sigma_r\ (N_r\ Z_r^2) ds$ (Kramers 1923)

where;

E = energy of probe electron.

Z = atomic number.

K = a constant.

r = an index running over all the constituent elements of both specimen or standard.

If we are able to compare a suitable standard with the specimen, equation 6 may be divided by 7 to yield;

8.

$$\frac{\text{(Characteristic counts from x / continuum counts)sp}}{\text{(Characteristic counts from x / continuum counts)std}} = \frac{(Nx / \Sigma\ Nr\ Zr^2)sp}{(Nx / \Sigma\ Nr\ Zr^2)std}$$

(Hall 1971).

where;

sp = specimen.

std = standard.

This quantitative procedure is known as continuum normalisation and, given a standard with a known concentration of element x in a matrix of known composition, it is possible to calculate the concentration of element x in an unknown specimen from the ratio of the characteristic counts generated by x and the continuum counts generated by the specimen (providing the composition of the specimen is either known or may be estimated).

Considering equation 5 again, that is;

$E = k (Z-z)^2$ (Warley 1997).

and also a modified form of equation 7 (see Warley 1997), viz.;

9. $W = KMt \Sigma(fa\ Za^2 / A_r(a))$ (Warley 1997).

where;

W = continuum intensity.

K = a constant.

Mt = total mass per unit area under analysis.

fa = mass fraction of element a.

Za = atomic number of element a.

$A_r(a)$ = atomic weight of element a.

The total mass per unit area under analysis is thus proportional to the continuum intensity. Mt may be calculated from the continuum intensity. Cx, the concentration of element x per total unit mass of specimen under analysis equates to;

10. $Cx = Mx/Mt = kx\ (Px/W)\ \Sigma(fa\ Za^2 / A_r(a))$ (Warley 1997).

where;

kx = elemental calibration constant.

Px = counts under the characteristic peak of element x.

Mx = mass of element x per unit area under analysed area.

The concentration of element x in mmol Kg^{-1} may be found from;

11. $$Cx\,(sp) = Cx\,(std) \bullet \frac{(Px/W)sp}{(Px/W)std} \bullet \frac{G\,(sp)}{G\,(std)}$$

(Warley 1997)

where;

Cx = elemental concentration (mmol Kg^{-1}).

$G = \Sigma(fa\ Za^2 / A_r(a))$.

G is a term commonly described as the G-factor. Equation 11 is equivalent to Hall's original equation (Hall 1971), see equation 12 below;

12. $Cx = Rx\ Ax\ Gx\ \Sigma m\ (C'\ Z^2/A)$ (Hall 1971).

where;

$$Rx = \frac{(\text{characteristic count for element x / continuum count})sp}{(\text{characteristic count for element x / continuum count})std}$$

A = atomic weight of x.

$G = Nx/\Sigma(NA)$ for x in the specimen.

$Cx = Nx\ Ax\ /\Sigma(NA)$.

$\Sigma m\ (C'\ Z^2/\ A) = G(sp) = \Sigma(fa\ Za^2/A_r(a))$.

The continuum normalisation method of quantification has the advantage that it estimates Cx independently of section thickness, and indeed Px/W is independent of section thickness up to 5μm (Hall 1979; Hall & Gupta 1983). A peripheral standard is unnecessary, and an absolute standard is required for only one element. In the Quantem-FLS program used during analysis in this work, the calibration constants of FST values equate to 1/kx, see equation 10. Some of the FST values used were calculated from relative detector efficiencies, see chapter four (Morgan *et al.* 1975).

Aside from mass loss from the specimen as a consequence of electron beam irradiation, the two most serious shortcomings of continuum-normalisation are problems associated with estimating G(sp), and the need for background corrections to the measured continuum counts. Since the value of G(sp) primarily depends upon elements undetectable using EDS (e.g. C, H, O), it is not possible to directly measure $\Sigma(fa\ Za^2/A_r(a))$ for biological specimens. Naturally, by using standards of known composition and which closely resembles that of the cell itself, G(std) may be calculated. Fortunately, G factors do not vary much in soft tissue; values of between 3.2 and 3.6 are usual (Hall 1979; Warley 1997). Commonly, a value of G is estimated and the equations solved for G iteratively until a sound approximation is reached (Hall & Gupta 1983; Warley 1979). Potentially more serious is the problem of extraneously generated X-ray quanta arising from sources other than the specimen and contributing to the total continuum counts. Such background counts derive mainly from the support film, the grid and the grid holder (Hall & Gupta 1983; Warley 1997). Use of low atomic number materials in the holder (beryllium in this study) in practice removes the requirement to compensate for holder-generated counts (Warley 1997). Corrections for the grid and film are essential however; grid corrections are made on the basis that in a selected area of the continuum, the counts generated by the grid are a constant fraction of the counts in the characteristic peak of a given element, The continuum generated by the film can be calculated, given;

13. $Wt = Wsp + Wg + Wf$ (Warley 1997)

where;
Wt = total continuum
Wsp = continuum produced by specimen
Wg = continuum produced by grid
Wf = continuum produced by film.

These corrections require spectra to be recorded from both the grid and support film before analysis.

The data derived from microanalysis using the quantification method described here has the units millimoles of element per kilogram dry weight of specimen (mmols Kg^{-1} d.w.) and as noted by Gupta this is of limited value to the

cellular physiologist, and can in some cases be misleading (Gupta 1993). A measure of the local dry mass of a section can be obtained by comparing analyses from the same area in the frozen-hydrated and frozen-dried states (Gupta & Hall 1979). The derived water fraction can then be used to calculate the elemental concentrations in mmols l^{-1} of water (Gupta & Hall 1979). Alternatively, the dry mass fraction can be estimated to yield approximate concentrations (Pivovarova *et al.* 1994a). Peripheral standards of known composition have also been successfully used to measure local dry mass (Zierold & Wessing 1990).

In summary, the quantitative X-ray microanalysis of frozen-dried cryosections in this study was effected using continuum normalisation. Whilst technical advances have made the analysis of small intracellular inclusions or even mitochondria possible (Pivovarova *et al.* 1994a), the limits of microanalysis have been well documented (Hall & Gupta 1983). Frustratingly, the low atomic number elements of most interest to the physiologist are the very elements around which there is the most doubt regarding the accuracy of their quantitation. It is therefore essential that all elemental data derived from X-ray microanalysis be interpreted with caution.

Appendix two.

Statistical analysis.

Normality of distribution.

(i) Rate of secretion results.

In order to ensure the appropriateness of the statistical testing to be used to analyse these data, the normality of distribution of the measurements recorded from *in vitro* Malpighian tubules was examined using Kolmogorov-Smirnov (KS) tests. The secretion rate data were separated into two separate groups since the experiments were performed with an interval of several months between them. These experiments used control saline during the two periods when the rate of secretion was measured. Table 1 shows the results of these experiments, with both male and female secretion rates combined. Experiment A was carried out between May and August 1996 and experiment B, between January and March 1997.

Table 1. Rates of fluid secretion and R2/R1% values from experiments A and B (male and female insects combined). All values are mean ± standard error of the mean.

Experiment	**Rate 1** (nl min^{-1})	**Rate 2** (nl min^{-1})	**R2/R1** %	**n**
A	1.763±0.174	1.248±0.118	82.60±8.23	47
B	1.810±0.174	1.462±0.247	87.17±9.35	31

To test the normality of distribution of the two experimental data sets, a series of KS tests were performed. Tables 2, 3 and 4 indicate the results of these tests. Data was considered to be non-normally distributed if $P<0.05$.

Table 2. KS normality test results for experiments A and B (male and female insects combined).

Experiment	Rate 1	Rate 2	R2/R1%	n
A	Z=1.304, P=0.067	Z=0.902, P=0.380	z=1.312, P=0.064	47
B	Z=0.820, P=0.513	Z=1.367, P=0.048*	z=0.591, P=0.877	31

Table 3. KS normality test results for experiment A males and females.

Sex	Rate 1	Rate 2	R2/R1%	n
Male	Z=0.751, P=0.067	Z=0.644, P=0.801	Z=1.060, P=0.211	29
Female	Z=0.997, P=0.273	Z=0.975, P=0.298	Z=0.909, P=0.381	18

Table 4. KS normality test results for experiment B males and females.

Sex	Rate 1	Rate 2	R2/R1%	n
Male	Z=0.416 P=0.995	Z=0.485, P=0.973	Z=0.749, P=0.630	14
Female	Z=0.715, P=0.687	Z=0.824 P=0.507	Z=0.641, P=0.805	17

Only in one case, experiment B, rate 2 (that is the mean male plus female R2), (see *, table 2), did the tests indicate a significant deviation from the expected normal distribution. Therefore, it was decided to regard both data sets as normally distributed since, in addition, no *a priori* could be found to account for a non-normal distribution. The remaining fluid secretion rate results were analysed assuming they were normally distributed.

(ii) Fluid cation composition results.

Control data was tested for normality using Kolmogorov-Smirnov tests. Table 5 show the results.

Table 5. KS normality test results for control $[K^+]$ and $[Na^+]$ in secreted fluid

Rate period	**$[K^+]$ mM**	**$[Na^+]$ mM**	**n**
Rate 1	Z=0.697, P=0.716	Z=1.220, P=0.102	9
Rate 2	Z=0.643, P=0.803	Z=0.534, P=0.938	9

None of the data showed a significant deviation from the expected normal distribution. The remaining cation composition data was therefore regarded as normally distributed.

(iii) Control X-ray microanalysis results.

In order to ensure that the appropriate statistical analysis of the elemental concentrations, the normality of the distribution for the X-ray microanalysis control data set of was also tested with Kolmogorov-Smirnov (KS) tests. Table 6 shows the results of these tests. As before, the data were regarded as significantly non-normally distributed if $P<0.05$. From these results, it was concluded that overall, the basement membrane data showed a substantial non-normal character, as did the Ca concentration values.

Table 6. KS normality test results for X-ray microanalysis control data set. * denotes significant difference (P<0.05),

Cell site	*n*	Elemental concentration (mmol Kg^{-1} dry weight)						
		K	Na	Cl	P	S	Mg	Ca
Basement membrane	202	Z=1.5202 P=0.0197*	Z=1.5204 P=0.0196*	Z=1.1638 P=0.1332	Z=2.7797 P=0.0000*	Z=1.6848 P=0.0068*	Z=1.0963 P=0.1807	Z=2.6171 P=0.0000*
Basal infoldings	84	Z=1.0564 P=0.2143	Z=1.3726 P=0.0462*	Z=1.1762 P=0.1257	Z=0.9946 P=0.2758	Z=1.8248 P=0.0260*	Z=1.5187 P=0.0198*	Z=1.7075 P=0.0059*
Basal cytoplasm	74	Z=0.6035 P=0.8596	Z=1.0096 P=0.2598	Z=1.4553 P=0.0289*	Z=0.5434 P=0.9292	Z=0.8093 P=0.5290	Z=0.8219 P=0.5090	Z=2.8611 P=0.0000*
Central cytoplasm	129	Z=0.9245 P=0.3597	Z=1.9464 P=0.0010*	Z=2.1028 P=0.0003*	Z=0.3501 P=0.9997	Z=0.7893 P=0.5609	Z=1.4940 P=0.0230*	Z=1.4911 P=0.0234*
Apical cytoplasm	24	Z=0.8757 P=0.4271	Z=0.6202 P=0.8365	Z=0.8367 P=0.4857	Z=0.5087 P=0.9581	Z=0.5225 P=0.9477	Z=1.0364 P=0.2230	Z=4.0308 P=0.0000*
Apical microvillar border	33	Z=0.6287 P=0.8241	Z=0.9873 P=0.2838	Z=1.0504 P=0.2198	Z=0.6854 P=0.7353	Z=0.5098 P=0.9573	Z=0.6092 P=0.8518	Z=1.7696 P=0.0380*

Bibliography.

Agar, A. W., R. H. Alderson and D. Chescoe (1974). Principles and practice of electron microscope operation. Practical methods in electron microscopy, volume 2. North-Holland, Amsterdam.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson (1989). Molecular biology of the cell. 2nd edition. Garland, London and New York.

Albrecht, F. O. (1953). The anatomy of the migratory locust. London.

Al-fifi, Z. I. A. (1997). "Biochemical, cytochemical localisation and physiological studies on Malpighian tubules of *Locusta migratoria* L.". (Durham University Ph.D. thesis).

Al-fifi, Z. I. A., J. H. Anstee and K. Bowler (1998a). The action of inhibitors of protein kinases on fluid and ion secretion by Malpighian tubules of *Locusta migratoria* L. Journal of Insect Physiology **44**: 973-980.

Al-fifi, Z. I. A., S. L. Marshall, D. Hyde, J. H. Anstee and K. Bowler (1998b). Characterisation of ATPases of apical membrane fractions from *Locusta migratoria* Malpighian tubules. Insect Biochemistry and Molecular Biology **28**: 201-211.

Anstee, J. H., P. Baldrick and K. Bowler (1986). Studies on ouabain-binding to (Na^+ + K^+)-ATPase from Malpighian tubules of the locust, *Locusta migratoria* L. Biochimica et Biophysica Acta **860**: 15-24.

Anstee, J. H. and D. M. Bell (1975). Relationship of to fluid production by Malpighian tubules of *Locusta migratoria*. Journal of Insect Physiology **21**: 1779-1784.

Anstee, J. H., D. M. Bell and H. Fathpour (1979). Fluid and cation secretion by the Malpighian tubules of *Locusta*. Journal of Insect Physiology **25**: 373-380.

Anstee, J. H., D. M. Bell and D. Hyde (1980). Some factors affecting Malpighian tubule fluid secretion and transepithelial potential in *Locusta migratoria* L. Experimentia **36**: 198-199.

Anstee, J. H. and K. Bowler (1979). Ouabain sensitivity of insect epithelial tissue. Comparative Biochemistry and Physiology **62A**: 763-769.

Aston, R. J. (1975). The role of adenosine 3':5'-cyclic monophosphate in relation to the diuretic hormone of *Rhodnius prolixus*. Journal of Insect Physiology **21**: 1873-1877.

Aston, R. J. (1979). Studies on the diuretic hormone of *Rhodnius prolixus*. Some observations on the purification and nature of the hormone and the dynamics of its release *in vitro*. Insect Biochemistry **9**: 163-176.

Audsley, N., G. M. Coast and D. A. Schooley (1993). The effects of *Manduca sexta* diuretic hormone on fluid transport by the Malpighian tubules and cryptonephric complex of *Manduca sexta*. Journal of Experimental Biology **178**: 231-243.

Baldrick, P. (1987). "Studies on ion movement in Malpighian tubules of Locusta migratoria L. with particular reference to electrical events". (Durham University PhD thesis).

Baldrick, P., D. Hyde and J. H. Anstee (1988). Microelectrode studies on Malpighian tubule cells of Locusta migratoria: effects of external ions and inhibitors. Journal of Insect Physiology **34**: 963-975.

Bell, D. M. (1977). "Studies on the Malpighian tubules of Locusta migratoria (R+F), with particular reference to the role of the Na^+ -K^+ -activated ATPase in fluid secretion". (Durham University PhD thesis).

Bell. D. M. and J. H. Anstee (1977). A study of the Malpighian tubules of *Locusta migratoria* by scanning and transmission electron microscopy. Micron. **8**: 123-134.

Bernard, C. (1878). Leçon sur les phénomènes de la vie communs aux animaux et aux végétaux. Paris, Ballière.

Berridge, M. J. (1965a). The physiology of excretion in the cotton stainer, *Dysdercus fasciatus* Signoret. I. Anatomy, water excretion and osmoregulation. Journal of Experimental Biology **43**: 511-521.

Berridge, M. J. (1965b). The physiology of excretion in the cotton stainer, *Dysdercus fasciatus* Signoret. III. Nitrogen excretion and excretory metabolism. Journal of Experimental Biology **43**: 535-552.

Berridge, M. J. (1966a). The physiology of excretion in the cotton stainer *Dysdercus fasciatus*, Signoret. IV. Hormonal control of excretion. Journal of Experimental Biology **44**: 553-566.

Berridge M. J. (1966b). Metabolic pathways of isolated Malpighian tubules of the blowfly functioning in an artificial medium. Journal of Insect Physiology **12**: 1523-1538.

Berridge M. J. (1968). Urine formation by the Malpighian tubules of *Calliphora*. I. Cations. Journal of Experimental Biology **48**: 159-174.

Berridge, M. J., B. D. Lindley and W. T. Prince (1975). Membrane permeability changes during stimulation of isolated salivary glands of *Calliphora* by 5-hydroxytryptamine. Journal of Physiology **244**: 549-567.

Berridge, M. J., B. D. Lindley and W. T. Prince (1976). Studies on the mechanism of fluid secretion by isolated salivary glands of *Calliphora*. Journal of Experimental Biology **64**: 311-322.

Berridge, M. J. and J. L. Oschman (1969). A structural basis for fluid secretion by Malpighian tubules. Tissue Cell **1**: 247-272.

Berridge, M. J. and N. G. Patel (1968). Insect salivary glands: stimulation of fluid secretion by 5-hydroxytryptamine and adenosine 3',5'-monophosphate . Science **162**: 462-463.

Berridge, M. J. and W. T. Prince (1972). Transepithelial potential changes during stimulation of isolated salivary glands with 5-hydroxytryptamine and cyclic AMP. Journal of Experimental Biology **56**: 139-153.

Berridge, M. J. and W. R. Schlue (1977). Ion-selective electrode studies on the effects of 5-hydroxytryptamine on the intracellular level of potassium in an insect salivary gland. Journal of Experimental Biology **72**: 203-216.

Bertram, G., L. Schleithoff, P. Zimmermann and A. Wessing (1991). Bafilomycin A_1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei*: is a vacuolar-type ATPase involved in ion and fluid secretion? Journal of Insect Physiology **37**: 201-209.

Beyenbach, K. W. (1993). Extracellular fluid homeostasis in insects? Pages 146-173 in K. W. Beyenbach, ed. Structure and function of primary messengers in invertebrates: insect diuretic and antidiuretic peptides. Molecular and comparative endocrinology, volume 12. Karger, Basel.

Beyenbach, K. W., A. Oviedo and D. J. Aneshansley (1993). Malpighian tubules of *Aedes aegypti*: five tubules, one function. Journal of Insect Physiology **39**: 639-648.

Blok-van Hoek, C. J. G., and J. F. M. Pinxter (1993). Recent developments in X-ray detectors and their relevance in biological microanalysis. Pages 3-18 in D. C. Sigee, A. J. Morgan, A. T. Sumner and A. Warley, eds. X-ray microanalysis in biology: experimental techniques and applications. Cambridge University Press.

Bowman, E. J., A. Siebers and K. Altendorf (1988). Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animals and plant cells. Proceedings of the National Academy of Sciences of the United States of America **85**: 7972-7976.

Bradley, T. J. (1985). The excretory system: structure and function. Pages 421-465 in G. A. Kerkut and L. I. Gilbert, eds. Comprehensive insect physiology, biochemistry and pharmacology. Volume 4. Regulation: digestion, nutrition, excretion. Pergamon, Oxford.

Bradley, T. J. (1989). Membrane dynamics in insect Malpighian tubules. American Journal of Physiology **257**: R967-R972.

Bradley , T. J. and P. Satir (1981). 5-Hydroxytryptamine -stimulated mitochondrial movement and microvillar growth in the Malpighian tubules of the insect *Rhodnius prolixus*. Journal of Cell Science **49**: 139-161.

Bradley, T. J., A. M. Stuart, P. Satir (1982). The ultrastructure of the larval Malpighian tubules of the saline-water mosquito, *Aedes taeniorhynchus*. Tissue Cell **14**: 759-773.

Brooks, E. J., A. J. Tousimis and L. S. Birks (1962). The distribution of calcium in the epiphyseal cartilage of the rat tibia measured with the electron probe x-ray microanalyser. Journal of Ultrastructural Research **7**: 56-60.

Burhop, E. H. S. (1969). The auger effect. Cambridge University Press.

Cantino, M. E., L. E. Wilkinson, M. K. Goddard and D. E. Johnson (1986). Beam induced mass loss in high resolution biological microanalysis. Journal of Microscopy **144**: 317-327.

Castaing, R. (1951). Ph.D. Thesis, University of Paris (O.N.E.R.A. publication number 55).

Cereijido, M., R. G. Contreras and L. Gonzalez-Mariscal (1989). Development and alteration of polarity. Annual Review of Physiology **51**: 785-795.

Chamberlin, M. E. and J. E. Philips (1980). Proline transport by locust Malpighian tubules. Amer. Zool. **20**: 938.

Chamberlin, M. E. and J. E. Philips (1982). Metabolic support of chloride-dependent short-circuit current across locust rectum. Journal of Experimental Biology **99**: 349-361.

Chandler, J. A. (1977). X-ray microanalysis in the electron microscope. Practical methods in electron microscopy, volume 5, part II. North-Holland, Amsterdam.

Chao, A. C. , D. F. Moffet and A. R. Koch (1991). Cytoplasmic pH and goblet cavity pH in the posterior midgut of the tobacco hornworm (*Manduca sexta*). Journal of Experimental Biology **155**: 403-414.

Charnley, A. K. (1975). "Some effects on the removal of the frontal ganglion on metabolism in *Locusta migratoria migratoroides* R. and F.".(Durham University PhD thesis).

Chen, C., L. Schleithof and J. P. Lokhandwala (1993). Relationship of polyphosphoinositide metabolism to the hormonal activation of the insect salivary gland by 5-hydroxytrptamine. Molecular Cell Endocrinology **36**: 37-42.

Cioffi, M. and M. G. Wolfsberger (1983). Isolation of separate apical, lateral and basal plasma membrane from cells of an insect epithelium. A procedure based on tissue organisation and ultrastructure. Tissue and Cell **15**: 781-803.

Clark, T. M. and J. H. Spring (1992). Excretion in the house cricket: role of second messengers in Malpighian tubule function. Canadian Journal of Zoology **70**: 1347-1353.

Clements, A. N. (1992). The biology of mosquitoes. Chapman and Hall, London.

Coast, G. M. (1988). Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus*, and their response to diuretic hormone. Physiological Entomology **13**: 381-391.

Coast, G. M. (1989). Stimulation of fluid secretion by single isolated Malpighian tubules of the house cricket *Acheta domesticus*. Physiological Entomology **14**: 21-30.

Coast, G. M. (1995). Synergism between diuretic peptides controlling ion and fluid transport in insect Malpighian tubules. Regulatory Peptides **57**: 283-296.

Coast, G. M. (1996). Neuropeptides implicated in the control of diuresis in insects. Peptides **17**: 327-336.

Coast, G. M. (1998). The influence of neuropeptides on Malpighian tubule writhing and its significance for excretion. Peptides **19**: 469-480.

Coast, G. M., O. Cusinato, I. Kay, G. J. Goldsworthy (1991). An evaluation of the role of cyclic AMP as an intracellular messenger in Malpighian tubules of the house cricket, *Acheta domesticus*. Journal of Insect Physiology **37**: 563-573.

Coast, G, M., G. M. Holman and R. J. Nachman (1990). The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket *Acheta domesticus*. Journal of Insect Physiology **36**: 481-488.

Coast, G, M. and I. Kay (1994). The effects of *Acheta*-diuretic peptide on isolated Malpighian tubules from the house cricket, *Acheta domesticus*. Journal of Experimental Biology **187**: 225-243.

Coast, G, M., I. Kay and C. H. Wheeler (1993). Diuretic peptides in the house cricket, *Acheta domesticus* (L.). Pages 38-66 in K. W. Beyenbach, ed. Structure and function of primary messengers in invertebrates: insect diuretic and antidiuretic peptides. Molecular and comparative endocrinology, volume 12. Karger, Basel.

Collier, K. A. and M. J. O'Donnell (1997). Analysis of epithelial transport by measurement of K^+, Cl^- and pH gradients in extracellular unstirred layers: ion secretion and reabsorption by Malpighian tubules of *Rhodnius prolixus*. Journal of Experimental Biology **200**: 1627-1638.

Cooper, P. D., G. G. E. Scudder and G. A. Quamme (1988). Changes in fluid and in secretion following stimulation in isolated Malpighian tubules of the water boatman *Cenocorixa blaisdelli* (Hung.) (Hemiptera, Corixidae). Journal of Insect Physiology **34**: 79-83.

Curran, P. F. (1960). Na, Cl and water transport by rat ileum *in vitro*. Journal of General Physiology **43**: 1137-1148.

Daly, H. V., J. T. Doyen and P. R. Ehrlich (1981). Introduction to insect biology and diversity. Magraw-Hill, London.

Davis, S.-A., G. R. Huesmann, S. H. P. Maddrell, M. J. O'Donnell, N. J. V. Skaer, J. A. T. Dow and N. J. Tublitz (1995). CAP_{2b}, a cardioacceleratory peptide is present in *Drosophila* and stimulates fluid secretion by cGMP. American journal of Physiology **269**: R1321-R1326.

Davis, S.-A., E. J. Stewart, G. R. Huesmann, N. J. V. Skaer, S. H. P. Maddrell, N. J. Tublitz and J. A. T. Dow (1997). Neuropeptide stimulation of the nitric oxide signalling pathway in *Drosophila melanogaster* Malpighian tubules. American Journal of Physiology **273**: R823-R827.

Del Rosario, A. D., H. X. Bui, M. Abdulla and J. S. Ross (1993). Sulphur-rich prostatic intraluminal granules. Human Pathology **24**: 1159-1167.

Diamond, J. M. and W. H. Bossert (1967). Standing-gradient osmotic flow: a mechanism for coupling water and solute transport in epithelia. Journal of General Physiology **50**: 2061-2083.

Dijkstra, S., A. Leyssens, E. Van Kerkhove, W. Zieske and P. Steels (1995). A cellular pathway for Cl^- during fluid secretion in ant Malpighian tubules: evidence from ion-selective microelectrode studies? Journal of Insect Physiology **41**: 695-703.

Dijkstra, S., E. Lohrmann, E, Van Kerkhove and R. Greger (1994). Characteristics of the luminal proton pump in Malpighian tubules of the ant. Renal Physiology and Biochemistry **17**: 27-39.

Donkin, J. and J.H. Anstee (1980). The effect of temperature on the ouabain-sensitivity of Na^+-K^+-activated ATPase and fluid secretion by the Malpighian tubules of *Locusta*. Experimentia **36**: 986-987.

Dörge, A., F. X. Beck, P. Weinecke and R. Rick (1989). Cl transport across the basolateral membrane of principal cells in frog skin. Miner. Electrolyte Metab. **15**:155-162.

Dörge, A., K. Gehring, W. Nagel and K. Thurau (1974). Intracellular Na^+-K^+ concentration of frog skin at different states of Na transport. Pages 337-349 in T. Hall, P. Echlin and R. Kaufman, eds. Microprobe analysis as applied to cells and tissues. Academic Press, London and New York.

Dörge, A. and R. Rick (1990). Studies of epithelial electrolyte transport by marker ions. Scanning Microscopy **4**: 449-455.

Dörge, A., R. Rick, F. Beck, and K. Thurau (1985). Cl transport across the basolateral membrane in frog skin epithelium. Pflügers Archiv **405**: suppl 1. S8-S11.

Dow, J. A. T., B. L. Gupta, and T. A. Hall (1981). Microprobe measurements of Na, K, Cl. P, S, Ca, Mg and water in frozen-hydrated sections of anterior caeca of the locust *Schistocerca gregaria*. Journal of Insect Physiology **27**: 629-639.

Dow, J. A. T., B. L. Gupta, T. A. Hall and W. R. Harvey (1984). X-ray microanalysis of elements in frozen-hydrated sections of an electrogenic K^+ transport system: the posterior midgut of tobacco hornworm (*Manduca sexta*) *in vivo* and *in vitro*. Journal of Membrane Biology **77**: 223-241,

Dow, J. A. T., S. H. P. Maddrell, S. -A. Davis, N. J. V. Skaer and K. Kaiser (1994). A novel role for the nitric oxide-GMP signalling pathway: the control of fluid secretion in *Drosophila*. American Journal of Physiology **266**: R1716-R1719.

Dow, J. A. T., S. H. P. Maddrell, A. Görtz, N. V. Skaer, S. Brogan and K. Kaiser (1994). The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. Journal of Experimental Biology **197**: 421-428.

Dröse, S. and K. Altendorf (1997). Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. Journal of Experimental Biology **200**: 1-8.

du Plessis, C. (1939). The incipient outbreaks of the Brown Locust in 1937-1938 with special reference to biometrical analysis. Scientific Bulletin of the Department of Agriculture, South Africa **209**: 69.

Duncumb, P. (1959). The X-ray scanning microanalyser. British Journal of Applied Physics **10**: 420-427.

Ehrenfeld, J, and U. Klein (1997). The key role of the H^+ V-ATPase in acid-base balance and Na^+ transport processes in frog skin. Journal of Experimental Biology **200**: 247-256.

Elder, H. Y., W. A. P. Nicholson, M. Mackenzie and A. D. Johnson (1997). A comparative look at microanalytical thin sample preparation problems in biology and material sciences. Proceedings of the Royal Microscopical Society **32**; 14-19.

Farquharson, P. A. (1974). A study of the Malpighian tubules of the pill millipede, *Glomeris marginata* (Villers) - II. The effect of variations in osmotic pressure and sodium and potassium concentrations on fluid production. Journal of Experimental Biology **60**: 29-39.

Fathpour, H., J. H. Anstee and D. Hyde (1983). The effect of Na^+, K^+, ouabain, amiloride and ethacrynic acid on transepithelial potential across Malpighian tubules of *Locusta*. Journal of Insect Physiology **29**: 773-778.

Fathpour, H., and D. L. Dahlman (1994). Effect of anions, acetazolamide, thiocyanate and amiloride on fluid secretion by the Malpighian tubules of *Locusta migratoria* L. Journal of Insect Physiology **40**: 1093-1099.

Fernandes, O. A., R. J. Wright, K. H. Baumgarten and Z. B. Mayo (1997). Use of rubidium to label *Lysiphlebus testaceipes* (Hymenoptera: Braconidae), a parasitoid of greenbugs (Homoptera: Aphididae), for dispersal studies. Environmental Entomology **26**: 1167-1172.

Fitzgerald, R., K. Keil and K. J. F. Heinrich (1968). Solid state energy dispersion spectrometer for electron microscope X-ray analysis. Science **159**: 528-530.

Fogg, K. E. (1990). "Studies on ion movement in Malpighian tubules of *Locusta migratoria* L. with particular reference to their endocrine control." (Durham University, Ph.D. thesis).

Fogg, K. E., D. Hyde and J. H. Anstee (1989). Microelectrode studies on Malpighian tubule cells of *Locusta*: effects of cyclic AMP, IBMX and corpora cardiaca extract. Journal of Insect Physiology **35**: 387-392.

Fogg, K. E., D. Hyde and J. H. Anstee (1990). Effects of corpora cardiaca extract on intracellular second messenger levels in Malpighian tubules of *Locusta migratoria*. Journal of Insect Physiology **36**: 383-389.

Fogg, K. E., D. Hyde and J. H. Anstee (1991). Studies on the subcellular distribution of $(Na^+ + K^+)$-ATPase, K^+-stimulated ATPase and HCO_3^- -stimulated ATPase activities in Malpighian tubules of *Locusta migratoria* L. Insect Biochemistry **21**: 749-758.

Fogg, K. E., D. Hyde and J. H. Anstee (1993). Effects of corpora cardiaca extract, furosemide and ion substitution on sodium and chloride flux in perfused Malpighian tubules of *Locusta*. Experimentia **49**: 296-299.

Fransson, L. (1987). Structure and function of cell associated proteoglycans. Trends in Biochemical Sciences **12**: 406-411.

Gallacher, D. V., Y. Marruyaman and O. H. Petersen (1984). Patch -clamp study of rubidium and potassium conductance in single ion channels from mammalian exocrine acini. Pflügers Arch. **401**: 361-367.

Garayoa, M., A. C. Villaro, U. Klein, B. Zimmermann, L. M. Montuenga, P. Sesma (1995). Immunocytochemical localisation of a vacuolar-type ATPase in Malpighian tubules of the ant *Formica polyctena*. Cell Tissue Research **282**: 343-350.

Geck, P., C. Pietrzyk, B.-C. Burckhardt, B. Pfeiffer and E. Heinz (1980). Electrically silent cotransport of Na^+, K^+ and Cl^- in Erlich cells. Biochimica et Biophysica Acta **600**: 432-447.

Gee, J. D. (1975a). Diuresis in the tsetse fly *Glossina austeni.* Journal of Experimental Biology **63**: 381-390.

Gee, J. D. (1975b). The control of diuresis in the tsetse fly *Glossina austeni* : a preliminary investigation of the diuretic hormone. Journal of Experimental Biology **63**: 391-401.

Gee, J. D. (1976a). Active transport of sodium by the Malpighian tubules of the tsetse fly *Glossina morsitans*. Journal of Experimental Biology **64**: 357-368.

Gee, J. D. (1976b). Fluid secretion by the Malpighian tubules of the tsetse fly *Glossina morsitans* : the effects of ouabain, ethacrynic acid and amiloride. Journal of Experimental Biology **65**: 323-332.

Giebisch, G. (1979). Principles of ion transport in kidney tubules. Pages 1-82 in G. M. O. Maloiy ed. Comparative physiology of osmoregulation in animals. Academic Press, London.

Gooding, R. H. (1975). Inhibition of diuresis in the tsetse fly (*Glossina morsitans*) by ouabain and acetazolamide. Experimentia **31**: 938-939.

Greger, R. (1985). Ion transport mechanisms in thick ascending limb of Henle's Loop of mammalian nephron. Physiological Reviews **65**: 760-797.

Grodowitz, M. J., A. B. Broce and K. J. Kramer (1987). Morphology and biochemical composition of mineralised granules from the Malpighian tubules of *Musca autumnalis* de Geer larvae (Diptera: Muscidae). Insect Biochemistry **17**: 335-345.

Gupta, B. L. (1979). The electron microprobe X-ray analysis of frozen-hydrated sections with new information on fluid transporting epithelia. Pages 375-408 in C. P. Lechne and R. R. Warner, eds. Academic Press, New York.

Gupta, B. L. (1989). The relationship of mucoid substances and ion and water transport, with new data on intestinal goblet cells and a model for gastric secretion. Pages 81-110 in E. Chantler and N. A. Radcliffe, eds. Mucus and related topics. Symposia of the Society of Experimental Biology number XLIII. The Company of Biologists, Cambridge.

Gupta, B. L. (1993). Electron probe -ray microanalysis of diffusible ions in cells and tissues from invertebrate animals. Pages 231-256 in D. C. Sigee, A. J. Morgan, A. T. Sumner and A. Warley, eds. X-ray microanalysis in biology: experimental techniques and applications. Cambridge University Press.

Gupta, B. L., M. J. Berrridge, T. A. Hall and R. B. Moreton (1978). Electron-probe and microelectrode studies of fluid secretion in the salivary glands of *Calliphora*. Journal of Experimental Biology **72**: 261-284.

Gupta, B. L. and T. A. Hall (1979). Quantitative electron probe X-ray microanalysis of electrolyte elements within epithelial tissue compartments. Federation Proceedings **38**: 114-153.

Gupta, B. L. and T. A. Hall (1981). Microprobe analysis of fluid-transporting epithelia: evidence for local osmosis and solute recycling. Pages 17-35 in H. H. Ussing, N. Bindslev, N. A. Lassen and O. Sten-Knudsen. Water transport across epithelia. Munkesgaard, Copenhagen.

Gupta, B. L., T. A. Hall, S. H. P. Maddrell and R. B. Moreton (1976). Distribution of ions in a fluid transporting epithelium determined by electron probe X-ray microanalysis. Nature **264**: 284-287.

Gupta, B. L., T. A. Hall and R. J. Naftalin (1978). Microprobe measurement of concentration and gradients of Na, K and Cl in epithelial cells and intracellular spaces of rabbit ileum. Nature **272**: 70-73.

Gupta, B L., B. J. Wall, J. L. Oschman and T. A. Hall (1980). Direct microprobe evidence of local concentration gradients and recycling of electrolytes during fluid absorption in the rectal papillae of *Calliphora*. Journal of Experimental Biology **88**: 21-48.

Haas, M. (1994). The Na-K-Cl cotransporters. American Journal of Physiology **267**: C869-C885.

Hall, T. A. (1971). The microprobe assay of chemical elements. Pages 157-275 in G. Oster, ed. Physical Techniques in Biological Research Volume I A. Academic Press, New York.

Hall, T. A. (1979). Problems of the continuum-normalisation method for the quantitative analysis of sections of soft tissue. Pages 185-208 in C. P. Lechne and R. R. Warner, eds. Microbeam Analysis in Biology. Academic Press, New York.

Hall, T. A. (1986). The history and the current status of biological-electron probe X-ray microanalysis. Micron and Microscopica Acta **17**: 91-100.

Hall, T. A. (1989). The history of electron probe microanalysis in biology. Pages 1-15 in K. Zierold and H. K. Hagler, eds. Electron probe X-ray microanalysis; applications in biology and medicine. Springer series in biophysics, volume 4. Springer - Verlag Berlin and Heidleberg.

Hall T. A. and B. L. Gupta (1974). Beam -induced loss of organic mass under electron microprobe conditions. Journal of Microscopy **100**: 177-188.

Hall, T. A. and B. L. Gupta (1982). Quantification for the X-ray microanalysis of cryosections. Journal of Microscopy **126**: 333-345.

Hall, T. A. and B. L. Gupta (1983). The localisation and assay of chemical elements by microprobe methods. Quarterly Review of Biophysics **16**: 279-339.

Hall, T. A. and B. L. Gupta (1986). EDS applications in biology. Pages 219-248 in D. C. Joy, A. D. Romig and J. I. Goldstein, eds. Principles of analytical electron microscopy. Plenum Publishing Corporation , New York.

Hamilton, A. G. (1955). Parthenogenesis in the Desert Locust (*Schistocerca gregaria* Forsk.) and its possible effect on the maintenance of the species. Proceedings of the Royal Entomological Society of London Series A, **30**: 103-114.

Hanrahan, J. and J. E. Philips (1983). Mechanism and control of salt absorption in locust rectum. American Journal of Physiology **244**: R131-R142.

Harvey, W. R. and H. Weiczorek (1997). Animal plasma membrane energiztion by chemiosmotic H^+-V-ATPases. Journal of Experimental Biology **200**: 203-216.

Hayes, T. K., T. L. Pannabecker, D. J. Hinkley, G. M. Holman, R. J. Nachman, D. H. Petzel and K. W. Beyenbach (1989). Leucokinins, a new family of ion transport inhibitors in insect Malpighian tubules. Life Sciences **44**: 1259-1266.

Hegarty, J. L., B. Zhang, T. L. Pannabecker, D. H. Petzel, M. D. Baustian and K. W. Beyenbach (1991). Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. American Journal of Physiology **261**: C521-C529.

Hichami, A., J. P. Anger, N. Allain, L. Vernhet, C. A. E. Martin and A. B. Legrand (1996). Stimulation of Rb^+ influx by bradykinin through $Na^+/K^+/Cl^-$ cotransport and Na^+/K^+-ATPase in NIH-3T3 fibroblasts. Life Sciences **59**: 1829-1837.

Hill, A. E. (1975a). Solute-solvent coupling in epithelia: a critical examination of the standing-gradient osmotic flow theory. Proceedings of the Royal Society of London Series B **190**: 99-114.

Hill, A. E. (1975b). Solute-solvent coupling in epithelia: and electro-osmotic theory of fluid transfer. Proceedings of the Royal Society of London Series B **190**: 115-134.

Hill, A. E. (1977). Mechanisms of solute-solvent coupling. Pages 183-214 in B. L. Gupta, R. B. Moreton, J. L. Oschman and B. J. Wall, eds. Transport of ions and water in animals. Academic Press, London and New York.

Hille, B. (1992). Ionic channels of excitable membranes. 2nd ed. Sinauer Associates, Massachuesetts.

Holman, G. M., B. J. Cook and R. J. Nachman (1986a). Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins. Comparative Biochemistry and Physiology **84C**: 205-211.

Holman, G. M., B. J. Cook and R. J. Nachman (1986b). Primary structure and synthesis of two additional neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins. Comparative Biochemistry and Physiology **84C**: 271-276.

Holman, G. M., B. J. Cook and R. J. Nachman (1987a). Isolation, primary structure and synthesis leucokinins V and VI: myotropic peptides of *Leucophaea maderae*. Comparative Biochemistry and Physiology **88C**: 27-30.

Holman, G. M., B. J. Cook and R. J. Nachman (1987b). Isolation, primary structure and synthesis leucokinins VII and VIII: the final members of this new family of cephalomyotropic peptides isolated from head extracts of *Leucophaea maderae*. Comparative Biochemistry and Physiology **88C**: 31-34.

Humbert, W. and P. Pévet (1995). Calcium concretions in the pineal gland of aged rats: an ultrastructural and microanalytical study of their biogenesis. Cell and Tissue Research **279**: 565-573.

Humphries, M. H. (1976). Inhibition of NaCl absorption from perfused rat ileum by furosemide. American Journal of Physiology **230**: 1517-1523.

Ichikawa, R., K. Hayashi and T. Nagata (1994). X-ray microanalysis of the secretory granules in the intestinal goblet cells of ageing mice. Medical Electron Microscopy **27**: 337-339.

Joly, P. and L. Joly (1953). Resultats de graffee de corpora allata chez *Locusta migratoria* L.. Ann. Sci. nat. zool. ser. **15**: p331-345.

Kalule-Sabiti, J. M. (1985). "A study on the effects of various inhibitors on the ATPase activity in, and fluid secretion by Malpighian tubules of *Locusta migratoria* L." . (Durham University Ph.D. thesis).

Kay, I., C. H. Wheeler, G. M. Coast, N. F. Totty, O. Custino, M. Patel and G. J. Goldsworthy (1991). Characterisation of a diuretic peptide from *Locusta migratoria*. Biol. Chem. Hoppe-Seyler **372**: 929-934.

Karnovsky, M. J. (1965). A formaldehyde-gluteraldhyde fixative of high osmolarity for use in electron microscopy. Journal of Cell Biology **27**: 137-138A.

Klein, U. (1992). The insect V-ATPase, a plasma membrane proton pump energising secondary active transport: immunological evidence for the occurrence of a V-ATPase in insect transporting epithelia. Journal of Experimental Biology **172**: 345-354.

Klein, U., G. Löffelmann and H. Wieczorek (1991). The midgut as a model system for insect K^+-transporting epithelia: immunocytochemical localisation of a vacuolar-type H^+ pump. Journal of Experimental Biology **161**: 61-75.

Kramers, H.A. (1923). On the theory of X-ray absorption and of the continuous X-ray spectrum. Philosophical Magazine **46**: 836-871.

Küppers, J. and I. Bunse (1996). A primary cation transport by a V-type ATPase of low specificity Journal of Experimental Biology **199**: 1327-1334.

Lechne, C. P. and R. R. Warner (1979). Electron probe analysis of liquid droplets. Pages 279-296 in C. P. Lechne and R. R. Warner, eds. Microbeam analysis in biology. Academic Press, New York.

Lever, J. D. and P. Duncumb (1961). The detection of intracellular ions in rat duodenal epithelium. Pages 278-286 in J. D. Boyd *et al.*, eds. Electron microscopy in anatomy. Edward Arnold, London.

Leyssens, A., S. Dijkstra, E. Van Kerkhove and P. Steels (1994). Mechanisms of K^+ uptake across the basal membrane of Malpighian tubules of *Formica polyctena*: the effect of ion and inhibitors. Journal of Experimental Biology **195**: 123-145.

Leyssens, A., P. Steels, E. Lohrmann, R. Weltens and E. Van Kerkhove (1992). Intrinsic regulation of K^+ transport in Malpighian tubules (*Formica*): electrophysiological evidence. Journal of Insect Physiology **38**: 431-446.

Leyssens, A., E. Van Kerkhove, S.-L. Zhang, R. Weltens and P. Steels (1993a). Measurement of intracellular and luminal K^+ concentration in a Malpighian tubule (*Formica*): estimate of basal and luminal electrochemical K^+ gradients. Journal of Insect Physiology **39**: 945-958.

Leyssens, A., S.-L. Zhang, E. Van Kerkhove and P. Steels (1993b). Both dinitrophenol and Ba^{2+} reduce KCl and fluid secretion in Malpighian tubules of *Formica polyctena*: the role of the apical H^+ and K^+ concentration gradient. Journal of Insect Physiology **39**: 1061-1073.

Liedtke, C. M. (1989). Regulation of chloride transport in epithelia. Annual Review of Physiology **51**: 143-160.

Longo, N., L. D. Griffin and L. J. Elsas (1991). A simple method of evaluation of Rb^+ transport and Na^+-K^+ pump stoichiometry in adherent cells. American Journal of Physiology **260**: C1341-C1346.

Maddrell, S. H. P (1962). A diuretic hormone in *Rhodnius prolixus* Stål. Nature **194**: 605-606.

Maddrell, S. H. P (1963). Excretion in blood-sucking bug, *Rhodnius prolixus* Stål. I. The control of diuresis. Journal of Experimental Biology **40**: 247-256.

Maddrell, S. H. P (1964a). Excretion in blood-sucking bug, *Rhodnius prolixus* Stål. II. The normal course of diuresis and the effect of temperature. Journal of Experimental Biology **41**: 163-176.

Maddrell, S. H. P (1964b). Excretion in blood-sucking bug, *Rhodnius prolixus* Stål. III. The control of the release of the diuretic hormone. Journal of Experimental Biology **41**: 459-472.

Maddrell, S. H. P (1969). Secretion by the Malpighian tubules of *Rhodnius*. The movements of ions and water. Journal of Experimental Biology **51**: 71-97.

Maddrell S. H. P. (1972). The functioning of insect Malpighian tubules. Pages 338-356 in L. Bolis, R.D. Keynes and W. Wildbrandt, eds. Role of membranes in secretory processes. North-Holland, Amsterdam.

Maddrell, S. H. P. (1977). Insect Malpighian tubules. Pages 541-569 in B. L. Gupta, R. B. Moreton, J. L. Oschman and B. J. Wall, eds. Transport of ions and water in animal tissues. Academic Press, London.

Maddrell, S. H. P (1981). The functional design of insect excretory system. Journal of Experimental Biology **90**:1-15.

Maddrell, S. H. P. and B. O. C. Gardiner (1976). Excretion of alkaloids by Malpighian tubules of insects. Journal of Experimental Biology **64**: 267-281.

Maddrell, S. H. P. and B. O. C. Gardiner (1980a). The retention of amino acids in the haemolymph during diuresis in *Rhodnius*. Journal of Experimental Biology **87**: 315-329.

Maddrell, S. H. P. and B. O. C. Gardiner (1980b). The permeability of the cuticular lining of the insect alimentary canal. Journal of Experimental Biology **85**: 227-237.

Maddrell, S. H. P., B. O. C. Gardiner, D. E. M. Pilcher and S. E. Reynolds (1974). Active transport by insect Malpighian tubules of acidic dyes and acylamides. Journal of Experimental Biology **61**: 357-377.

Maddrell, S. H. P., W. S. Herman, R. W. Farndale and J. A. Riegel (1993). Synergism of hormones controlling epithelial fluid transport in an insect. Journal of Experimental Biology **174**: 65-80.

Maddrell, S. H. P., W. S. Herman, R. L. Mooney and J. A. Overton (1991). 5-Hydroxytryptamine: a second diuretic hormone in *Rhodnius*. Journal of Experimental Biology **156**: 557-566.

Maddrell, S. H. P. and S. Klunsuwan (1973). Fluid secretion by *in vitro* preparations of the Malpighian tubules of the desert locust *Schistocerca gregaria*. Journal of Insect Physiology **19**: 1369-1376.

Maddrell, S. H. P. and M. J. O'Donnell (1992). Insect Malpighian tubules: V-ATPase action in ion and fluid transport. Journal of Experimental Biology **172**: 417-429.

Maddrell, S. H. P. and J. E. Philips (1975). Active transport of sulphate ions by the Malpighian tubules of the larvae of the mosquito *Aedes aegypti*. Journal of Experimental Biology **62**:367-378.

Maddrell, S. H. P., D. E. M. Pilcher and B. O. C. Gardiner (1969). Stimulatory effect of 5-hydroxytryptamine (serotonin) on secretion by Malpighian tubules of insects. Nature, **222**: 784-785.

Maddrell, S. H. P., D. E. M. Pilcher and B. O. C. Gardiner (1971). Pharmacology of the Malpighian tubules of Rhodnius and Carausius: the structure-activity relationship of tryptamine analogues and the role of cyclic AMP. Journal of Experimental Biology **54**: 779-804.

Marshall, A. T. (1980). Principles and instrumentation. Pages 1-64 in M. A. Hayat, ed. X-ray microanalysis in biology. Macmillan, London.

Marshall, A. T., P. Cooper, G. D. Ripon and A. E. Patek (1993). Ion and fluid secretion by different segments of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*. Journal of Experimental Biology **177**: 1-22.

Marshall, S. L. (1995). "Studies on the mechanisms of ion and fluid secretion by Malpighian tubules of *Locusta migratoria* L.." (Durham University, Ph.D. thesis).

Moffet, D. F. and A. R. Koch (1988a). Electrophysiology of K^+ transport by midgut epithelium of lepidopteran insect larvae I. The transbasal electrochemical gradient. Journal of Experimental Biology **135**: 25-38.

Moffet, D. F. and A. R. Koch (1988b). Electrophysiology of K^+ transport by midgut epithelium of lepidopteran insect larvae. II. The transapical electrochemical gradients. Journal of Experimental Biology **135**: 39-45.

Mordue, W. (1969). Hormonal control of Malpighian tube and rectal function in the desert locust, *Schistocerca gregaria*. Journal of Insect Physiology **15**: 273-285.

Mordue, W. and G. J. Goldsworthy (1969). The physiological effects of corpus cardiacum extracts in locusts. Gen. Comp. Endocr. **12**: 360-369.

Moreton, R. B., P. Echlin, B. L. Gupta, T. A. Hall and T. Weis-Foch (1974). Preparation of frozen hydrated tissue sections for X-ray microanalysis in the scanning electron microscope. Nature **247**: 113-115.

Morgan, A. J. (1980). Preparation of specimens. Pages 65-165 in M. A. Hayat, ed. X-ray microanalysis in biology. Macmillan, London.

Morgan, A. J. (1985). X-ray microanalysis in electron microscopy for biologists. Royal Microscopical Society Microscopy Handbooks, No. 5, Oxford University Press, Oxford.

Morgan, A. J., T. W Davies and D. A. Erasmus (1975). Analysis of droplets from isoatomic solutions as a means of calibrating a transmission electron analytical microscope (TEAM). Journal of Microscopy **104**: 270-280.

Morgan, P. J. and W. Mordue (1981). Stimulated fluid secretion is sodium dependant in the Malpighian tubules of *Locusta migratoria*. Journal of Insect Physiology 27: 271-279.

Morgan, P. J. and W. Morgan (1983a). Separation and characterisation of diuretic hormone from corpus cardiacum of *Locusta*. Comparative Biochemistry and Physiology B **75**: 75-80.

Morgan, P. J. and W. Mordue (1983b). Electrochemical gradients across *Locusta* Malpighian tubules. Journal of Comparative Physiology **151**: 175-183,

Morgan, P. J. and W. Morgan (1984a). Diuretic hormone: another peptide with widespread distribution within the CNS. Physiological Entomology **9**: 197-206.

Morgan, P. J. and W. Morgan (1984b). 5-Hydroxytryptamine stimulated fluid secretion in locust Malpighian tubules independently of cAMP. Comparative Biochemistry and Physiology C **79**: 305-310

Morgan, P. J. and W. Morgan (1985). Cyclic AMP and locust diuretic hormone action: hormone induced changes in cAMP levels offers a novel method for detecting biological activity of uncharacterised peptide. Insect Biochemistry **15**: 247-257.

Morgan, P. J., K. J. Siegert and W. Morgan (1987). Preliminary characterisation of locust diuretic peptide (DP-1) and another corpus cardiacum peptide (LCCP). Insect Biochemistry **17**: 383-388.

Mörk, A.-C., A. V. Euler and G. M Roomans (1992). Effect of chronic treatment with diuretics on mouse liver: a morphological and microanalytical investigation. Scanning Microscopy **6**: 1147-1153.

Mörk, A.-C., A. V. Euler, G. M Roomans and A. Ring (1997). cAMP-induced chloride transport in NCL-SG3 sweat gland cells. Acta Physiologica Scandinavica **157**: 21-32.

Mörk, A.-C. and G. M. Roomans (1993). X-ray microanalysis of cAMP induced ion transport in NCL-SG3 sweat gland cells. Scanning Microscopy **7**: 1233-1240.

Moseley, H.J.G. (1913). On the high frequency spectra of the elements. Philosophical Magazine **26**: 1024-1034.

Nässel, D. R., P.C.C.M. Passier, K. Elekes, H. Dircksen, H. G. B. Vullings and R. Cantera (1995). Evidence that locustatachykinin I is involved in release of adipokinetic hormone from locust corpora cardiaca. Regulatory Peptides **57**: 297-310.

Nicolson, S. W. and S. A. Hanrahan (1986). Diuresis in a desert beetle? Hormonal control of the Malpighian tubules of *Onymacris plana*. Journal of Comparative Physiology B **156**: 407-413.

Nicolson, S. W., and L. C. Isaacson (1987). Transepithelial and intracellular potential in isolated Malpighian tubules of tenebrionid beetle. American Journal of Physiology **252**: F645-F653.

Nicolson, S. W., and L. C. Isaacson (1996). Mechanism of enhanced secretion in the warmed Malpighian tubules of the tsetse fly *Glossina morsitans morsitans*. Journal of Insect Physiology **42**: 1027-1033.

Nuñez, J.A. (1956). Untersuchungen über die Regelung des Wasserhaushaltes bei *Anisotarsus cupripennis* Germ. Z. vergl. Physiol. **38**:341-354.

O'Donnell, M. J., J. A. T. Dow, G. R. Huesmann, N. J. Tublitz and S. H. P. Maddrell (1996). Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. Journal of Experimental Biology **199**: 1163-1175.

O'Donnell, M. J. and S. H. P. Maddrell (1984). Secretion by the Malpighian tubules of *Rhodnius prolixus* Stål : electrical events. Journal of Experimental Biology **110**: 275-290.

O'Grady, S. M., M. W. Musch and M. Field (1986). Stoichiometry and ion affinities of the Na-K-Cl cotransport system in the intestine of the winter flounder (*Pseudopleuronectes americanus*). Journal of Membrane Biology **91**: 33-41.

O'Grady, S. M., H. C. Palfrey and M. Field (1987). Characteristics and functions of Na-K-Cl cotransport in epithelial tissues. American Journal of Physiology **253**: C177-C192.

O'Neill, K. M., S. A. Woods and D. A. Streett (1997). Grasshopper (Orthoptera: Acridadae) foraging on grasshopper feces: observational and rubidium-labelling studies. Environmental Entomology **26**: 1224-1231.

Oschman, J. L. and M. J. Berridge (1970). Structural and functional aspects of salivary gland secretion in *Calliphora*. Tissue and Cell **2**: 281-310.

Palfrey, H. C., P. W. Feit and P. Greengard (1980). cAMP-stimulated cation cotransport in avian erythrocytes: inhibition by loop diuretics. American Journal of Physiology **238**: C139-C148.

Palfrey, H. and C. P. Greengard (1981). Hormone sensitive ion transport systems in erythrocytes as models for epithelial ion pathways. Annals of the New York Academy of Sciences **372**: 291-308.

Pannabecker, T. L., T. K. Hayes and K. W. Beyenbach (1993). Regulation of epithelial shunt conductance by the peptide leucokinin. Journal of Membrane Biology **132**: 63-76.

Parsons, D., D. J. Bellotto, W. W. Schulz, M. Buja and H. K. Hagler (1984). Towards routine cryoultramicrotomy. EMSA Bulletin **14**: 49-60.

Patel, M., J.-S. Chung, I. Kay, A. I. Mallet, C. R. Gibbon, K. S. J. Thompson, J. P. Bacon and G. M. Coast (1994). Localisation of *Locusta*-DP in the locust central nervous system and haemolymph satisfies initial hormonal criteria. Peptides **15**: 591-602.

Patel, M., T. K. Hayes and G, M. Coast (1995). Evidence for the hormonal function of a CRF-related diuretic peptide (*Locusta*-DP) in *Locusta migratoria*. Journal of Experimental Biology **198**: 793-804.

Peacock, A. J. (1975). "Studies on the excretion and neuroendocrine system of some orthopteran insects, with particular reference to *Jamaicana flava* (Caudell)". (Durham University PhD thesis).

Peacock, A. J., K. Bowler and J. H. Anstee (1972). Demonstration of a Na^+-K^+-Mg^{2+} dependant ATPase in a preparation from hindgut Malpighian tubules of two species of insect. Experimentia **28**: 901-902.

Pérez-Cornejo, P. and T. Begenisich (1994). The multi-ion nature of the pore in *Shaker* K^+ channels. Biophysical Journal **66**: 1929-1938.

Petzel, D. H. , M. M. Berg and K. W. Beyenbach (1987). Hormone-controlled, cAMP-mediated diuresis in the yellow fever mosquito. American Journal of Physiology **253**: R701-R711.

Petzel, D. H. H. Hagedorn and K. W. Beyenbach (1985). Preliminary isolation of mosquito naturetic factor. American Journal of Physiology **249**: R379-R386.

Petzel, D. H. H. Hagedorn and K. W. Beyenbach (1986). Peptide nature of two mosquito naturetic factors. American Journal of Physiology **250**: R328-R332.

Petzel, D. H., E. Melvim and J. Knezetic (1998). Cloning of the Na-H exchanger in mosquito Malpighian tubules. FASEB Journal **12**: A1025.

Phillips, J. (1964a). Rectal absorption in the desert locust, *Schistocerca gregaria* Forskål. I. Water. Journal of Experimental Biology **41**: 15-38.

Phillips, J. (1964b). Rectal absorption in the desert locust, *Schistocerca gregaria* Forskål. II. Sodium, potassium and chloride. Journal of Experimental Biology **41**: 39-67.

Phillips, J. (1964c). Rectal absorption in the desert locust, *Schistocerca gregaria* Forskål. III. The nature of the excretory process. Journal of Experimental Biology **41**: 68-80.

Phillips, J. (1981). Comparative physiology of insect renal function. American Journal of Physiology **241**: R241-R257.

Phillips, J., N. Audsley, R. Lechleitner, B. Thomson, J. Meredith and M. Chamberlin (1988). Some major transport mechanisms of insect absorptive epithelia. Comparative Biochemistry and Physiology **90A**: 643-650.

Pietrantonio, P. V. and S. S. Gill (1995). Immunolocalisation of the 17kDa vacuolar H^+-ATPase subunit c in *Heliothis virescens* midgut and Malpighian tubules with an anti-peptide antibody. Journal of Experimental Biology **198**: 2609-2618.

Pivovarova, N. , J. H. Anstee and K. Bowler (1993). The use of Rb in a study of cell function in Malpighian tubules of locusts by X-ray microanalysis. Cell Biology International **17**: 971-972.

Pivovarova, N. , J. H. Anstee and K. Bowler (1994b). An X-ray microanalytical study on the effects of ouabain and N-ethyl maleimide on the elemental concentrations in Malpighian tubule cells of *Locusta*. Scanning Microscopy Supplement **8**: 37-45.

Pivovarova, N. , S. L. Marshall, J. H. Anstee and K. Bowler (1994a). An X-ray microanalytical study of *Locusta* Malpighian tubule cell function using rubidium. American. Journal of Physiology **266**: R1551-1561.

Plawner, L., T. L. Pannabecker, S. Laufer, M. D. Baustian and K. W. Beyenbach (1991). Control of diuresis in the yellow fever mosquito Aedes aegypti : evidence for similar mechanisms in the male and female. Journal of Insect Physiology **37**: 119-128.

Prince, W. T. and M. J. Berridge (1972). The effects of 5-hydroxytryptamine and cyclic AMP on the potential profile across isolated salivary glands, Journal of Experimental Biology **56**: 323-333.

Proux, J. (1991). Lack of responsiveness of Malpighian tubules to the AVP-like insect diuretic hormone on migratory locusts infected with the protozoan *Malameba locustae*. Journal of Invertebrate Pathology **58**: 353-361.

Quamme, G. A. (1988). X-ray analysis of biological fluids: an update. Scanning Electron Microscopy **2**: 2195-2205.

Rafaeli, A., M. Pines, P. S. Sterns and S. W. Applebaum (1984). Diuretic hormone stimulated synthesis and excretion of cyclic-AMP by locust Malpighian tubules. Gen. Comp. Endocr. **54**: 35-42.

Quinlan, M. C. and M. J. O' Donnell (1998). Anti-diuresis in the blood feeding insect *Rhodnius prolixus* Stål: antagonistic actions of cAMP and cGMP and the role of organic acid transport. Journla of Insect Physiology **44**: 561-568.

Rafaeli, A. and W. Mordue (1982). The responses of the Malpighian tubules of *Locusta* to hormones and other stimulants. Gen. Comp. Endocr. **46**:130-135.

Rafaeli-Bernstein, A. and W. Mordue (1978). The transport of the cardiac glycoside ouabain by the Malpighian tubules of *Zonocerus variegatus*. Physiological Entomology **3**: 59-63.

Rafaeli-Bernstein, A. and W. Mordue (1979). The effects of phlorizin, phloretin, and ouabain on the reabsorption of glucose by the Malpighian tubules of *Locusta migratoria migratoroides* Journal of Insect Physiology **25**: 241-247.

Ramsay, J. A. (1953). Active transport of potassium by the Malpighian tubules of insects. Journal of Experimental Biology **30**: 358-369.

Ramsay, J. A. (1954). Active transport of water by the Malpighian tubules of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae). Journal of Experimental Biology **31**: 104-113.

Ramsay, J. A. (1956). Excretion by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae): calcium, magnesium, chloride, phosphate and hydrogen ions. Journal of Experimental Biology **32**: 697-708.

Ramsay, J. A. (1958). Excretion by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). Journal of Experimental Biology **32**: 183-199.

Ramsay, J. A., R. H. J. Brown and S. W. H. W. Falloon (1953). Simultaneous determination of sodium and potassium in small volumes of fluid by flame photometry. Journal of Experimental Biology 30: 1-17.

Reed, S. J. B. (1993). Electron microprobe analysis. Cambridge University Press, Cambridge.

Regan, J. D. (1994). Expression cloning of an insect diuretic hormone receptor. Journal of Biological Chemistry. **269**: 9-12.

Regan, J. D. (1995). Molecular cloning of a putative Na^+-K^+-$2Cl^-$ cotransporter from the Malpighian tubules of the tobacco hornworm, *Manduca sexta*. Insect Biochemistry and Molecular Biology **8**: 875-880.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain. Journal of Cell Biology **17**: 208-212.

Rick, R., A. Dörge, R. Bauer, K, Gehring and K. Thurau (1979). Quantification of electrolytes in freeze-dried cryosections by electron microprobe analysis. Scanning Electron Microscopy **2**: 619-626.

Rick, R. and W. Schratt (1989). Distribution of ions and water in epithelial cells and tissues. Pages 213-223 in K. Zierold and H. H. Hagler, eds. Electron probe microanalysis: applications in biology and medicine. Springer-Verlag, Berlin.

Robertson, A. J., D. Rivers, G. Nagelschmidt and P. Duncumb (1961). Stannosis. Benign pnuemoconiosis due to tin dioxide Lancet 1089-1093.

Roinel, N. (1988). Quantitative X-ray analysis of biological fluids: the microdroplet technique. Journal of Electron Microscope Technique **9**: 45-56.

Roinel, N. and C. de Rouffignac (1993). X-ray microanalysis of biological fluids; applications to investigations in renal physiology. Pages 175-194 in D. C. Sigee, A. J. Morgan, A. T. Sumner and A. Warley, eds. X-ray microanalysis in biology: experimental techniques and applications. Cambridge University Press.

Roomans, G. M. (1983). Standards for X-ray microanalysis of biological specimens. Scanning Electron Microscopy/1979/**II**: 649-657.

Roomans, G. M. and L.A. Seveus (1977). Preparation of thin cryosectioned standards for quantitative microprobe analysis. Journal of Submicroscopic Cytology **9**: 31-35.

Roos, N. and A. J. Morgan (1985). Aminoplastic standards for electron probe X-ray microanalysis (EXPMA) of ultrathin frozen-dried cryosections. Journal of Microscopy **140**: RP3-RP4.

Russell, V. E. W., U. Klein M. Reuveni, D. D. Spaeth, M. G. Wolfsberger, and W. R. Harvey (1992). Antibodies to mammalian and plant V-ATPases cross react with the V-ATPase of insect cation-transport plasma membranes. Journal of Experimental Biology 166: 131-143.

Saga, K., and K. Sato (1989). Electron probe X-ray microanalysis of cellular ions in the eccrine secretory coil cells during methacholine stimulation. Journal of Membrane Biology **107**: 13-24.

Sasaki, S., I. Nakagaki, H. Kondo and S. Hori (1996). Changes in elemental concentrations induced by agonist in pig pancreatic acinar cells. Pflügers Archiv **432**: 538-545.

Saubermann, A. J., C. M. Castiglia and M. C. Foster (1992). Preferential uptake of rubidium from extracellular space by glial cells compared to neurones in leech ganglia. Brain Research **577**: 64-72.

Saubermann, A. J., D. C. Dobyan, V. L. Scheid and R. E. Bulger (1986). Rat renal papilla: comparison of two techniques for X-ray microanalysis. Kidney International **29**: 675-681.

Savage, A. A. (1956). The development of the Malpighian tubules of *Schistocerca gregaria* (Orthoptera). Quarterly Journal of Microscopical Sciences **97**: 599-615.

Sawyer, D. B. and K. W. Beyenbach (1985). Dibutyryl cAMP increases basolateral sodium conductance of mosquito Malpighian tubules. American Journal of Physiology **248**: R339-R345.

Schirrmans, K. and W. Zeiske (1991). Properties of basal K^+ channels in K^+-transporting *Manduca sexta* caterpillar midgut. Pflügers Archiv **418 (Suppl. 1)**: R74.

Schofield, R. M. S, J. H. Postlethwaite and H. Lefevre (1997). MeV-ion microprobe analyses of whole *Drosophila* suggest that zinc and copper accumulation is regulated storage no deposit excretion. Journal of Experimental Biology **200**: 3235-3243.

Schoofs, L., J. V. Broek and A. De Loof (1993). Mini-review: the myotropic peptides of *Locusta migratoria*: structures, distribution, functions and receptors. Insect Biochemistry and Molecular Biology **23:** 859-881.

Schoofs, L., G. M. Holman, T. K. Hayes, J. P. Kochansky, R. J. Nachman and A. De Loof (1991). Locustatachykinin III and IV: two additional insect neuropeptides with homology to peptides of the vertebrate tachykinin family. Regulatory Peptides **31**: 199-212.

Schoofs, L., G. M. Holman, T. K. Hayes, R. J. Nachman and A. De Loof (1990). Locustatachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. FEBS Letters **261**: 397-401.

Schoofs, L., G. M. Holman, P. Proost, J. Van Damme, T. K. Hayes and A. De Loof (1992). Locustakinin, a novel myotropic peptide from *Locusta migratoria*, isolation, primary structure and synthesis. Regulatory Peptides **37**: 49-57.

Schoofs, L., D. Veelaert, J. V. Broeck and A. De Loof (1996). Immunocytochemical distribution of locustamyoinhibiting peptide (Lom-MIP) in the nervous system of *Locusta migratoria*. Regulatory Peptides **63**: 171-179.

Schweikel H., U. Klein, M. Schindlbeck and H. Weiczorek (1989). A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. Journal of Biological Chemistry **264**: 11136-11142.

Servala, V. L. and K. G. Davey (1993). Juvenile hormone-dependant phosphorylation of a 100kDa polypeptide is mediated by protein kinase C in follicle cells of *Rhodnius prolixus*. Invertebrate Reproductive Development **23**: 189-193.

Shennan, D. B. (1989). Evidence for furosemide-sensitive Na^+-K^+-$2Cl^-$ co-transport in lactating rat mammary tissue. Quarterly Journal of Experimental Physiology **74**: 927-938.

Sigee, D. C. (1997). Environmental scanning electron microscopy and X-ray microanalysis of biological materials. Pages 129-148 in "Modern developments and applications in microbeam analysis". Conference proceedings from the 5th European Workshop of the European Microbeam Analysis Society.

Sigee, D. C., A. J. Morgan, A. T. Sumner and A. Warley (1993). X-ray microanalysis in biology: experimental techniques and applications. Cambridge University Press.

Skaer, H. (1989). Cell division in Malpighian tubule development in *D. melanogaster* is regulated by a single tip cell. Nature **342**: 566-569.

Skaer, H. (1993). The alimentary canal. Pages 941-1012 in M. Bate and A. Martinez Arias, eds. The development of *Drosophila melanogaster*. Cold Spring Harbor Press, New York.

Skou, J. C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across cell membranes. Physiological Reviews **45**: 596-617.

Sokal, R. R. and F. J. Rohlf (1981). Biometry. The principals and practice of statistics in biological research. 2nd edition. Freedman, New York.

Somlyo, A. P. (1985). Cell calcium measurement with electron probe and electron energy loss analysis. Cell Calcium **6**: 197-212.

Somlyo, A. V. and J. Silcox (1979). Cryoultramicrotomy for electron probe analysis. Pages 535-550 in C.P. Lechne and R.R. Warner, eds. Microbeam Analysis in Biology. Academic Press, New York.

Spring, J. H. (1990). Endocrine regulation of diuresis in insects. Journal of Insect Physiology **36**: 13-22.

Spring, J. H. and S. R. Hazelton (1987). Excretion in the house cricket (*Acheta domesticus*): stimulation of diuresis by tissue homogenates. Journal of Experimental Biology **129**: 63-81.

Steinbrecht, R. A. and M. Müller (1987). Freeze-substitution and freeze drying. Pages 149-176 in R. A. Steinbrecht abd K. Zierold, eds. Cryotechniques in biological electron microscopy. Springer-Verlag, Berlin and Heidleberg.

Stobbart, R. H. (1977). The control of diuresis following a bloodmeal in females of the yellow fever mosquito *Aedes aegypti*. Journal of Experimental Biology **69**: 53-85.

Taylor, H. H. (1971a). Water and solute transport by the Malpighian tubules of the stick insect *Carausius morosus*. The normal ultrastructure of the type I cell. Z. Zellforsch. Mik. Anat. **118**: 333-368.

Taylor, H. H. (1971b). The fine structure of the type II cells in the Malpighian tubules of the stick insect *Carausius morosus*. Z. Zellforsch. Mik. Anat. **122**: 411-424.

Thompson, K. S. J., R. C. Rayne, C. R. Gibbon, S. T. May, M. Patel, G. M. Coast and J. P. Bacon (1995). Cellular co-localisation of diuretic peptides in locusts: A potent control mechanism. Peptides **16**: 95-104.

Tousimis, A. J. and I. Adler (1963). Electron probe X-ray microanalyser study of copper within Descemet's membrane in Wilson's disease. Journal of Histochemistry and Cytochemistry **11**: 40-47.

Ussing, H. H. (1982). Volume regulation of frog skin epithelium. Acta Physiologica Scandinavia. **114**: 363-369.

Uvarov, B. (1966). Grasshoppers and locusts. A handbook of general acridology. Cambridge University Press.

Van Kerkhove, E. (1994). Cellular mechanisms of salt secretion by the Malpighian tubules of insects. Belgian Journal of Zoology **124**: 73-90.

Vietinghoff, U. (1967). Neurohormonal control of renal function in *Carausius morosus.* Gen. comp. Endocr. **9**: 503.

von Zglinicki, T. (1993). Radiation damage and low temperature X-ray microanalysis. Pages 117-131 in D. C. Sigee, A. J. Morgan, A. T. Sumner and A. Warley, eds. X-ray microanalysis in biology. Experimental techniques and applications. Cambridge University Press.

Wall, B. J. (1970). Water and solute uptake by rectal pads of *Periplanta americana.* American Journal of Physiology **218**: 1208-1215.

Wall, B. J. (1971). Local osmotic gradients in the rectal pads of an insect. Federation. Proceedings. **30**:42-48.

Wall, B. J., J. I. Oschman and B. A. Schmidt (1975). Morphology and function of Malpighian tubules and associated structures in the cockroach, *Periplanta americana.* Journal of Morphology **146**: 265-306.

Wall, B. J. and C. L. Ralph (1964). Evidence for the hormonal regulation of Malpighian tubule excretion in the insect *Periplanta americana* L. Gen. Comp. Endocr. **4**: 452-456.

Wang, S., A. B. Rubenfeld, T. K. Hayes and K. W. Beyenbach (1996). Leucokinin increases paracellular permeability in insect Malpighian tubules. Journal of Experimental Biology **199**: 2537-2542.

Warley, A. (1990). Standards for the application of X-ray microanalysis to biological specimens. Journal of Microscopy **157**: 135-147.

Warley, A. (1997). X-ray microanalysis for biologists. Practical Methods in Electron Microscopy, Volume16. Portland Press, London.

Warley, A. and B. L. Gupta (1991). Quantitative biological X-ray microanalysis. Pages 243-281 in J. Harris, ed. Electron microscopy in biology: A practical approach. IRL Press, Oxford.

Watt, F., J. P. Lansberg G. W. Grime and B. McDonald (1993). Proton probe microanalysis in biology: general principles and applications for the study of Alzheimer's disease. Pages 62-79 in D. C. Sigee, A. J. Morgan, A. T. Sumner and A. Warley, eds. X-ray microanalysis in biology: experimental techniques and applications. Cambridge University Press.

Weber-von Grotthuss, E., F. Hevert, U. Atzbacher and A. Wessing (1974). Influence of ouabain on Na^+ and K^+ concentration in haemolymph of *Drosophila hydei* and appearance of Malpighian tubules. Journal of Insect Physiology **20**: 1411-1420.

Weiczorek, H. (1992). The insect V-ATPase, a plasma membrane proton pump energising secondary active transport: molecular analysis of electrogenic potassium transport in the tobacco hornworm midgut. Journal of Experimental Biology **172**: 335-343.

Weiczorek, H., M. Putzenlechner, W. Zeiske and U. Klein (1991). A vacuolar-type proton pump energises K^+/H^+ antiport in an animal plasma membrane. Journal of Biological Chemistry **266**: 15340-15347.

Weiczorek, H., S. Weerth, M. Schnindlbeck and U. Klein (1989). A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membrane from tobacco hornworm midgut. Journal of Biological Chemistry 264: 11143-11148.

Weiczorek, H., M. G. Wolfsberger, M. Cioffi and W. R. Harvey (1986). Cation-stimulated ATPase activity in purified plasma membrane from tobacco hornworm midgut. Biochimie et Biophysica Acta **857**: 271-281.

Weltens, R., A. Leyssens, S.-L. Zhang, E. Lohrmann, P. Steels and E. Van Kerkhove (1992). Unmasking of the apical electrogenic H^+ pump in isolated Malpighian tubules (*Formica polyctena*) by the use of barium. Cellular and Physiological Biochemistry **2**: 101-116.

Wessing, A. and K. Zierold (1992). Metal salt feeding caused alteration of concretions in *Drosophila* Malpighian tubules as revealed by X-ray microanalysis. Journal of Insect Physiology **38**: 623-632.

Wessing, A. and K. Zierold (1993). Heterogeneous distribution of elements in the larval Malpighian tubules of *Drosophila hydei*: X-ray microanalysis of freeze-dried cryosections. Cell and Tissue Research **272**: 491-497.

Wessing, A. and K. Zierold (1996). The importance of the Golgi complex for epithelial ion transport in *Drosophila* Malpighian tubules, studied by electron microscopy, cytochemistry and X-ray microanalysis. European Journal of Cell Biology **69**: 116-127.

Wessing, A., G. Bertram and K. Zierold (1986). Ion transport and intracellular activity of ions in Malpighian tubules of *Drosophila hydei.* Zool. Beitr. N. F. **30**: 297-314.

Wessing, A., G. Bertram and K. Zierold (1993). Effects of bafilomycin A_1 and amiloride on the apical potassium and proton gradients in *Drosophila* Malpighian tubules studied by X-ray microanalysis and microelectrode measurements. Journal of Comparative Physiology B **163**: 452-462.

Wessing, A., K. Zierold and G. Bertram (1997). Carbonic anhydrase supports electrolyte transport in *Drosophila* Malpighian tubules. Evidence by X-ray microanalysis of cryosections. Journal of Insect Physiology 43: 17-28.

Wessing, A., K. Zierold and F. Hevert (1992). Two types of concretions in *Drosophila* Malpighian tubules as revealed by X-ray microanalysis: a study on urine formation. Journal of Insect Physiology **38**: 543-554.

Wessing, A., K. Zierold and D. Schäfer (1988). Intracellular storage of sodium and magnesium in *Drosophila* Malpighian tubules. X-ray microanalysis of native cryosections. European Journal of Cell Biology **47**; 1-6.

Whiteford, M. L., D. L. Bovell, J. D. Pediani, G. L. Smith, H. Y. Elder, C. M. Lee and S. M. Wilson (1993). The regulation of chloride permeability by ATP in a cell line NCL-SG3 established from human sweat gland epithelium. Journal of Physiology **467**: 104P.

Wigglesworth, V. B. (1931a). The physiology of excretion in a blood-sucking insect *Rhodnius prolixus* (Hemiptera, Reduviidae). I. Composition of the urine. Journal of Experimental Biology **8**: 411-427.

Wigglesworth, V. B. (1931b). The physiology of excretion in a blood-sucking insect *Rhodnius prolixus* (Hemiptera, Reduviidae). II. Anatomy and histology of the excretory system. Journal of Experimental Biology **8**: 428-442.

Wigglesworth, V. B. (1931c). The physiology of excretion in a blood-sucking insect *Rhodnius prolixus* (Hemiptera, Reduviidae). III. The mechanism of uric acid excretion. Journal of Experimental Biology **8**: 443-451.

Wigglesworth, V. B. and M. M. Saltpeter (1962). Histology of the Malpighian tubules in *Rhodnius prolixus* Stål (Hemiptera). Journal of Insect Physiology **8**: 299-307.

Wilkins, A. S. (1995). Singling out the tip cell of the Malpighian tubules-lessons from neurogenesis. Bioessays **17**: 199-201.

Williams, J. C. and K. W. Beyenbach (1983). Differential effects of secretagogues on Na and K secretion in the Malpighian tubules of *Aedes aegypti* (L.). Journal of Comparative Physiology B. **149**: 511-517.

Williams, J. C. and K. W. Beyenbach (1984). Differential effects of secretagogues on the electrophysiology of the Malpighian tubules of the yellow fever mosquito. Journal of Comparative Physiology B. **154**: 301-309.

Wilson, S. M., D. L. Bovell, H. Y. Elder, D. M. Jenkinson and J. D. Pediani (1990). The effect of removing external sodium upon the control of potassium ($^{86}Rb^{+}$) permeability in the isolated human sweat gland. Experimental Physiology **75**: 649-656.

Xu, J. -C., C. Lyle, T. T. Zhu, J. A. Payne, E. Benz Jr. and B. Forbush III (1994). Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. Proceedings of National Academy of Sciences of the United States of America **91**:2201-2205.

Zeiske, W. (1992). Insect ion homeostasis. Journal of Experimental Biology **172**: 323-334.

Zhang, S. L., A. Leyssens, E. Van Kerkhove. R. Weltens, W. Van Driessche and P. Steels (1994). Electrophysiological evidence for the presence of apical H^+-ATPase in Malpighian tubules of Formica polyctena. Intracellular and luminal pH measurements. Pflügers Archiv **426**: 3-4.

Zhang, A. L. and G. M. Roomans (1997). Ion transport in cultured pig tracheal submucosal gland acinar cells studied by X-ray microanalysis. European Respiratory Journal **10**: 2204-2209.

Zhong, C., Y. Ling, Z. Yu, W. Yu, K. Fujimoto and K. Ogawa (1995). Atrial specific granules-an intracellular calcium store. Acta Histochem. Cytochem. **28**: 507-511.

Zierold, K. (1985). Contrast in biological cryosections in scanning transmission electron microscopy. Journal of Microscopy **140**: 65-71.

Zierold, K. (1988). X-ray microanalysis of freeze dried and frozen-hydrated cryosections. Journal of Electron Microscopy Techniques **9**: 65-82.

Zierold, K. (1990). Cryofixation methods for ion localisation in cells by electron probe microanalysis; a review. Journal of Microscopy **161**:357-366.

Zierold, K. (1993). Rapid freezing techniques for biological electron probe microanalysis. Pages 101-116 in D. C. Sigee, A. J. Morgan A. T. Sumner and A. Warley, eds. X-ray Microanalysis in Biology: Experimental Techniques and Applications. Cambridge University Press, Cambridge.

Zierold K. and A. Wessing (1990). Mass dense vacuoles in *Drosophila* Malpighian tubules contain zinc, not sodium. A reinvestigation by X-ray microanalysis of cryosections. European Journal of Cell Biology **53**: 222-226.